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Studies in the Genus *Eremascus*

I. The Rediscovery of *Eremascus albus* Eidam and Some New Observations concerning its Life-history and Cytology

BY

C. E. HARROLD

(*Botany School, Cambridge*)

With Plates III and IV, and ten Figures in the Text

I. INTRODUCTION

IN 1883 Eidam reported the discovery of a new genus of ascomycete moulds in which naked asci, formed by fusion of two equal cells borne on short entwined hyphae, were produced singly throughout the mycelium. The fungus, which grew with other contaminants in a discarded flask of malt extract, was named *Eremascus albus*, the type species of a new genus. No cytological or physiological investigations were reported in Eidam's paper, and the fungus was lost before further work could be undertaken. Later, Stoppel (1907) described a second species, *E. fertilis*, found on the surface of apple-jelly, which closely resembled Eidam's fungus save for differences in spore shape and reproductive details. She investigated its cytology, as did also Guilliermond (1909), whose work extended our knowledge of parthenogenesis in this species. No other literature dealing with experimental work on *Eremascus* had appeared, nor had Eidam's species been found again, when the present investigation began. The studies reported here are concerned with new information about the life-history and cytology of *E. albus*; a second paper dealing with the environmental factors which control the occurrence of this rare species is in course of preparation.

In December 1944 Professor F. T. Brooks, of the Cambridge Botany School, received from Sir Frank Engledow a tin of mustard powder which was caked together and permeated throughout by a practically pure culture of a white mould; the mustard had been in home storage for a long time, perhaps for years. Examination showed the presence of numerous 8-spored asci freely borne without any kind of investment. Preliminary cultures (isolate 1) demonstrated that the asci were formed by the fusion of two equal cells at the end of short entwined hyphae and that the asci occurred over the whole mycelium. He was reminded of Eidam's figures of *E. albus* (1883) and provisionally identified the mould as this species. He then asked friends for other old samples of household mustard. Two, provided by Sir Rowland Biffen, also contained a similar white mould, but one was so heavily contaminated by Penicillia and Aspergilli that a pure culture could not be obtained. The other

[Annals of Botany, N.S. Vol. XIV, No. 54, April, 1950.]

sample gave isolate 2, which agreed entirely with isolate 1. In addition, another white mould (isolate 3), producing chains of arthrospores, was isolated from these mustard samples, but at the time it was uncertain whether it was a distinct fungus or a phase in the life-history of what was presumed to be *E. albus*. Isolate 3 never produced asci and was subsequently shown to have no connexion with *E. albus*; it awaits identification and will not be considered in detail in this paper.

Isolates 1 and 2 grew very slowly on ordinary media such as Dox's and 2 per cent. malt agar. Mr. J. Bean (Professor Brooks's technical assistant) found that they grew appreciably better on malt agar to which 40 per cent. sucrose had been added; the cultures were then maintained on this medium, which will be referred to as malt-sucrose agar.

In July 1945 Professor Brooks kindly handed all the cultures to me for further investigation, it being suggested that isolates 1 and 2 were actually the long-lost *E. albus*. It was soon found that the addition of 0·5 per cent. Difco yeast extract to the malt-sucrose medium enhanced growth and sporulation; this medium will be referred to as malt-sucrose-yeast agar. The present study aims at providing a more complete account of the development and structure of *E. albus*, and of the environmental factors which control its growth. It is influenced by the osmotic pressure of the medium and by relative humidity in an unusual fashion, being intolerant of saturated conditions; data concerning this aspect of the investigation will shortly be presented in a second paper.

II. FURTHER ISOLATIONS OF *EREMASCUS ALBUS*

Three new isolates of *E. albus* were obtained during the routine examination of substrates for the possible presence of this apparently rare species. In December 1947 several colonies of *E. albus* were discovered on the surface of mincemeat (isolate 4), and in April 1948 a further isolate was obtained from stored greengage jam where the mould was present as a pale yellowish mat on the surface (isolate 5). The white colonies on the mincemeat gradually turned pale yellow as they aged, and this fact, coupled with the yellow colour on the jam, provides an interesting comparison with the development of a characteristic yellow colour on malt-sucrose-yeast agar which is described later.

In a series of papers on mould deterioration of feeding-stuffs in relation to the humidity of storage, Snow et al. (1944) and Snow (1945) noted that a few moulds, especially those of the *Aspergillus glaucus* group, would develop at relative humidities of 75 per cent. and below. Although growth was very slow, a few moulds developed on locust beans stored for two years at 65 per cent. R.H. Samples of household mustard were set up at low humidities (76 and 80 per cent. R.H.) and examined at intervals for the possible appearance of *E. albus* among any moulds developing on them. Experiments had shown that isolates 1 and 3 could grow at these humidities, and since Eidam's record in 1883 was the only one for the species before the present isolations were made, the demonstration of its occurrence in household mustard as a possible contaminant was thought to be of interest. The jars containing the mustard samples (taken from locally purchased tins of mustard powder) were incubated at 25° C. over salt solutions giving the required humidities and examined at monthly intervals. The dominant mould was a white-spored *Aspergillus*, but in addition to this, chains of conidia resembling those associated with isolate 3 and asci of *E. albus* were also seen after about 3 months in one of the six samples examined. Development was allowed to continue for 12 months when isolations from portions of the mustard bearing the greatest development of asci were made. Finally, pure cultures were obtained of the two fungi corresponding to isolate 1 (i.e. *E. albus*) and isolate 3 respectively. This culture of *E. albus* is isolate 6.

Pure lines of each isolate were set up from single ascospores isolated by means of a micropipette mounted on a Zeiss micromanipulator. All strains proved to be homothallic. The details of the isolations of *E. albus* may be summarized as follows:

Substrate.	Isolation and locality.	Number.
Drying malt extract	E. Eidam, 1883, Germany	Type species; lost
Household mustard powder	F. T. Brooks, 1944, Cambridge	Isolate 1
	,, ,, ,, ,,	Isolate 2
	C. E. Harrold, 1948, Cambridge	Isolate 6
Mincemeat	,, ,, ,, London	Isolate 4
Greengage jam	,, ,, ,, Cambridge	Isolate 5

III. THE LIFE-HISTORY OF *EREMASCUS ALBUS*

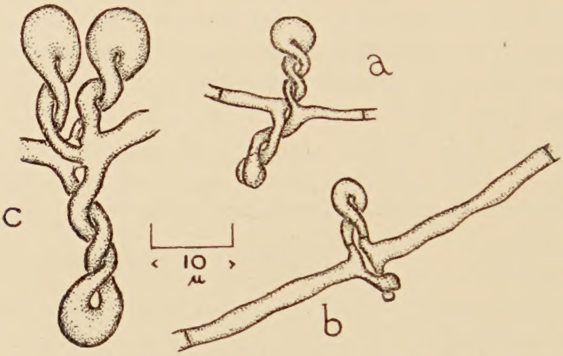
a. Slide culture technique

It was necessary to observe the details of ascus formation in the present isolates in order to confirm their identification as *E. albus*, and a slide culture technique was used for this purpose.

The method of Talbot (1945) was modified since an abundance of spores was not available. With a flamed wire loop, a drop of sterile water was placed on a clean flamed slide, and a sterile needle, previously touched on the surface of a sporing culture of *E. albus*, was stirred into this drop to produce a spore suspension. A drop of malt-sucrose agar was placed on a flamed coverslip and this was carefully lowered on to the inoculum drop. The two drops combined, holding the coverslip firmly to the slide with the seeded culture medium spread between the two. The size of the water and agar drops was adjusted so that approximately half the area beneath the coverslip was occupied by the culture medium. Such slide cultures were then incubated in sterile Petri dishes at 25° C. on glass triangles over a 16 per cent. saline solution (approximately 90 per cent. R.H.). This procedure was necessary since the use of water produced saturated conditions which prevent the development of *E. albus*; this effect will be considered in a later paper. The fungus develops and fruits rapidly in such slide cultures. Hyphae and asci stand out clearly in a colourless matrix of agar, and septa in the hyphae are clearly visible. Observations were carried out at intervals to ascertain the time required for the production of ascospores after the inception of ascus-formation, and the structures involved could be photographed *in situ* at different stages of development. Contamination of such cultures was very rare even though subjected to periodical examination under laboratory conditions.

b. Germination

Ascospores on malt-sucrose agar in a hanging-drop, over 16 per cent. saline solution at 25° C., germinated within 18 hours. Eidam (1883) and Stoppel (1907) stated that germination in their species required 2 days, but did not record their experimental conditions. Since temperature and humidity variations markedly influence germination time (thus germination of ascospores of the present isolates took 4 days at 25° C. and 80 per cent. R.H.), we cannot regard the discrepancy as important.



TEXT-FIG. 1. *E. albus*: coiling and fusion of gametangia during ascus formation.

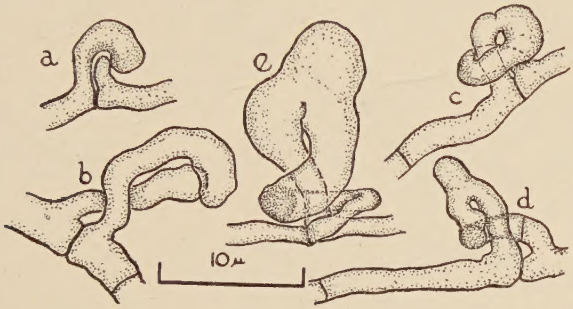
Germination is brought about by the splitting of the exosporium at one end of the almost spherical ascospore. The endosporium protrudes at this point to form a swollen vesicle from which one or more germ-tubes develop (Pl. III, *a*). Eidam (1883) records the production of germ-tubes at each end of the ascospore, but this has only rarely been seen in my preparations.

c. The normal process of ascus formation in Eremascus albus

Ascospores are delimited inside a spherical ascus produced by the fusion of two coiling gametangia (Text-fig. 1; Pl. III, *b*, *c*, *d*). The number of coils varies but is commonly more than one, whereas in *E. fertilis* coiling rarely occurs between the gametangia. Groups of gametangia are common and they arise from different points around the same part of a hypha, as in Eidam's original figures for *E. albus* (his Fig. 1, *c*). The two gametangia of a pair always arise from adjacent cells close to the dividing septum.

Eidam (1883) stated that the apices of the coiled gametangia were delimited from the basal regions before their fusion; in *E. fertilis*, however, fusion takes place without such prior delimitation of fusion cells (Stoppel, 1907). The position in the present isolates is intermediate since both conditions have been recorded. Thus, out of 23 asci in which development was closely watched 14 instances of fusion without previous septation of the gametangia were noted (Text-fig. 1, *a*, *c*), while only in two gametangial coils was there indubitable septation prior to fusion (Text-fig. 1, *b*). Examination of the present isolates revealed structures comparable with all those figured by Eidam (1883) except for his figure of 8-spored asci produced singly at the ends of hyphae. The variation in coiling and septation of the gametangia is shown in Text-fig. 1. Pl. III, *d* illustrates three stages in ascus formation; the gametangial fusion, the swelling of the ascus at the point of fusion, and finally, the delimitation of the ascospores.

The original description of *E. albus* contains no estimate of the time required for ascospore-formation. Periodical observation of selected asci in slide cultures showed that the process of ascus formation, from the production of gametangia to the delimitation of the ascospores, takes 5–6 days at 22·5° C. The ascospores are not dispersed by any special mechanism; the ascus wall disintegrates leaving eight ascospores adhering together in a cluster; such clusters may persist for long periods.



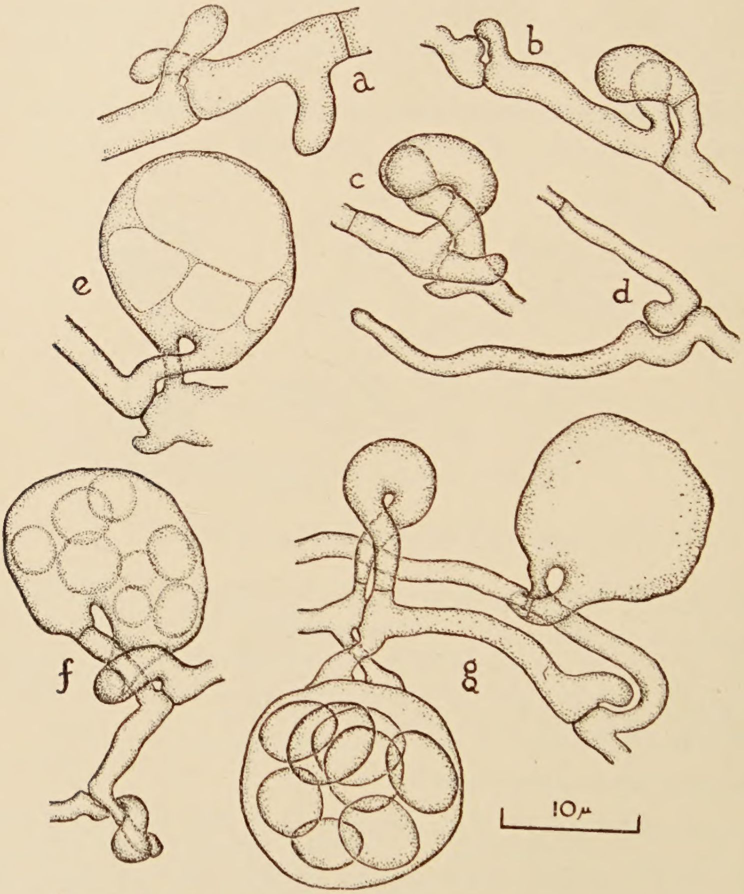
TEXT-FIG. 2. *E. albus*: anisogamous fusions.

d. Variations in ascus formation

The normal process of ascus formation in *E. albus* involves an isogamous fusion between two gametangia, but many variations have been observed which are of interest in discussing the relationships between *E. albus* and other members of the Endomycetales and Hemiascomycetes.

1. *Anisogamous fusion.* Instead of producing two equal gametangia the pairs of cells responsible for gametangium formation often put out unequal gametangia which, nevertheless, fuse to produce normal asci. Text-fig. 2, *a* illustrates the extreme case in which a larger gametangium completely dominates its smaller partner, while Text-fig. 2, *b*, *c*, *d* show similar anisogamy in which a certain degree of gametangial coiling is also present. Text-fig. 2, *e* illustrates ascus formation involving a smaller centrally placed gametangium about which is coiled a larger one, a situation highly suggestive of the condition found in Gymnoascus and Ctenomyces among the Gymnoascaceae. Usually the position of ascus formation in both isogamous and anisogamous

fusions is at the region of fusion and not displaced to one side as in Text-fig. 2, *c*. Such displacement of ascus formation coupled with anisogamy, as found exceptionally in *E. albus*, suggests the manner in which the more specialized antheridia and ascogonia in the higher Ascomycetes may have evolved from an isogamous ancestry.

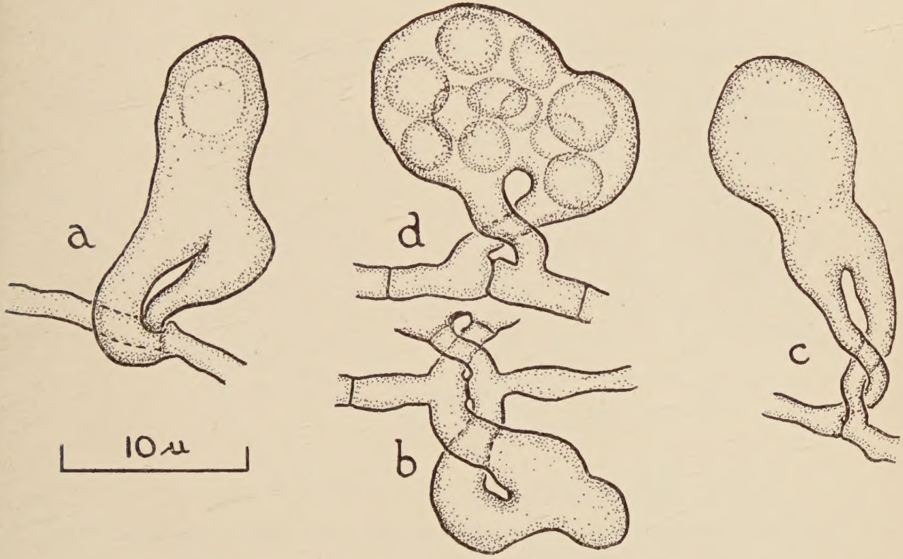


TEXT-FIG. 3. *E. albus*: anomalous development of gametangia.

2. *Anomalous development of gametangia.* While it is common for many pairs of gametangia to arise from the same two parent cells (Text-fig. 1, *c*), they usually do so close to each other at the septum separating the two cells. Occasionally, however, a cell which has produced a gametangium at one end will develop another at its opposite end without any septation of the cell taking place. Text-fig. 3, *b* shows the beginning of such a condition, while in Text-fig. 3, *f* we see a more advanced stage in which a cell having already produced a gametangium involved in ascus formation has given rise to another at its opposite end, which in turn is undergoing copulation. It may be remarked

that in the situation recorded in Text-fig. 3, *f* the smaller partners of both fusions arise from the same cell.

Sometimes a pair of cells having already undergone gametangial formation develops new outgrowths which appear to have no sexual function (Text-fig. 3, *e*). Text-fig. 3, *a* shows a pair of cells which have produced structures resembling gametangia but in which coiling ceased early and the gametangia grew on as sterile hyphae; a similar situation is recorded in Text-fig. 3, *c*, where the smaller gametangia lost their functions and underwent no subsequent coiling or fusion.



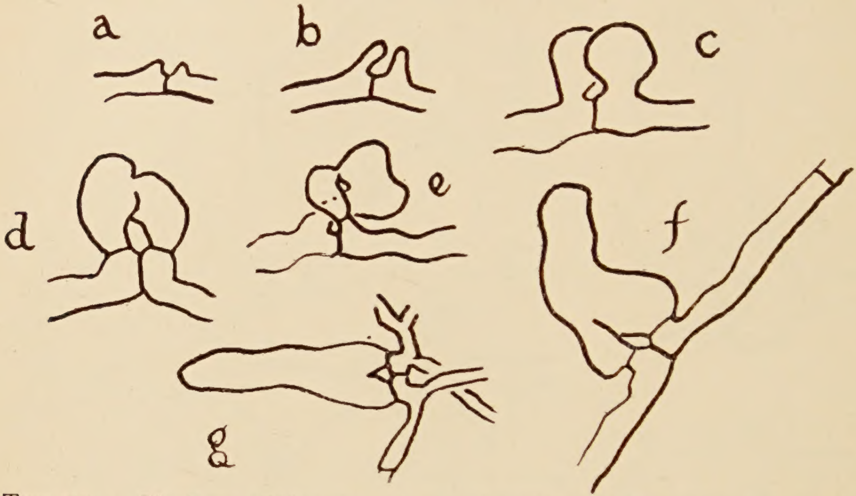
Text-fig. 4. *E. albus*: variations in ascus shape.

Rarely, one member of a gametangial pair ceases to function as a gametangium and continues its growth as a hypha (Text-fig. 3, *d*). Text-fig. 3, *g* shows an example of a gametangium which had developed into a hypha and later produced gametangia and an ascus.

3. *Variations in ascus shape and ascospore number.* Eidam (1883) made no mention of any departure from the normal spherical ascus shape nor of any variation in the normal number of eight ascospores per ascus. Examination of both Petri dish and slide cultures disclosed the occurrence of certain variations bearing an interesting resemblance to the sexual process in *Dipodascus albidus* (Lagerheim, 1892; Juel, 1902). In *D. albidus* there is a fusion of two multinucleate gametangia leading to the production of an elongate multispored ascus (Text-fig. 5). Fusions in *E. albus* reminiscent of certain illustrations in Lagerheim's paper are shown in Text-fig. 4 (cf. the formation of an elongate ascus in *E. albus*, Text-fig. 4, *a*, *b*, *c* with Text-fig. 5, *f*). Cytological preparations of these unusual structures in *E. albus* were not obtained, but they are

presumably uninucleate, whereas those of *Dipodascus albidus* are multinucleate.

Sometimes there is a reduction in the number of ascospores per ascus, as shown in Text-fig. 6, *c*. This is probably due to abortion of some of the eight spores originally formed. Text-fig. 6, *a* shows six fully formed ascospores with two others incompletely developed. Continued division of the eight nuclei resulting from the normal division of the fusion nucleus in the ascus leads to the formation of many ascospores above the normal complement of eight. Such ascospores, as shown in Text-fig. 6, *b*, are smaller than usual but they germinate and otherwise behave normally. Similar reduction and increase in the number of ascospores per ascus were also observed in cultures of *E. fertilis*. Ascospore formation in the multispored asci of the species of Eremascus is by free-cell formation typical of the Ascomycetes and not by continuous cleavage characteristic of the sporangia of Phycomycetes.



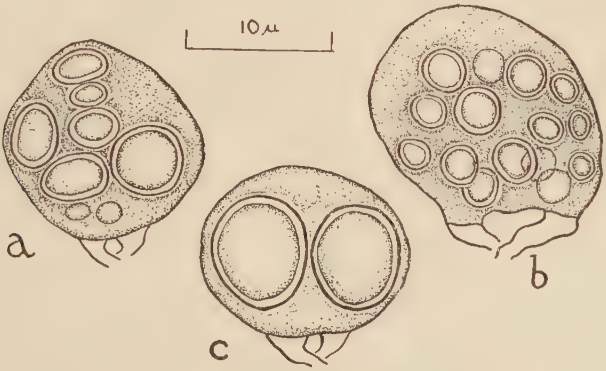
TEXT-FIG. 5. *Dipodascus albidus*: stages in ascus-formation. (After Lagerheim, 1892.)

e. The Thallospores of Eremascus albus

The original accounts of *E. albus* and *E. fertilis* describe no spore form other than the ascospore; in the present isolates, however, another structure referred to below as a thallospore (Mason 1933, pp. 6–7) was constantly found.

During thallospore development the tip of a hypha becomes swollen and assumes a spherical shape (Text-fig. 7, *a–c*), after which it is cut off by a septum from the hypha (Text-fig. 7, *d*, *e*). The significance of a double septum which not uncommonly occurs is unknown. Later the wall of the thallospore thickens (Text-fig. 7, *f–l*), and not infrequently the thallospore shape is elongate (Text-fig. 7, *j*, *k*). Intercalary thallospores are also found (Text-fig. 7, *l*). Production of thallospores is sometimes associated with the gametangia, one member of a coiling pair growing out considerably and cutting off a thallospore at its apex. Most remarkable, however, is the production of such spores at the apices of germ-tubes in many instances. Small isolated portions of agar are often present in the slide cultures referred to above, and in these ascospores often germinate and form thallospores directly with no development of mycelium; similar development has also been observed in hanging-drop cultures (Pl. III, *f*). These spores are cut off within 7 days of spore germination.

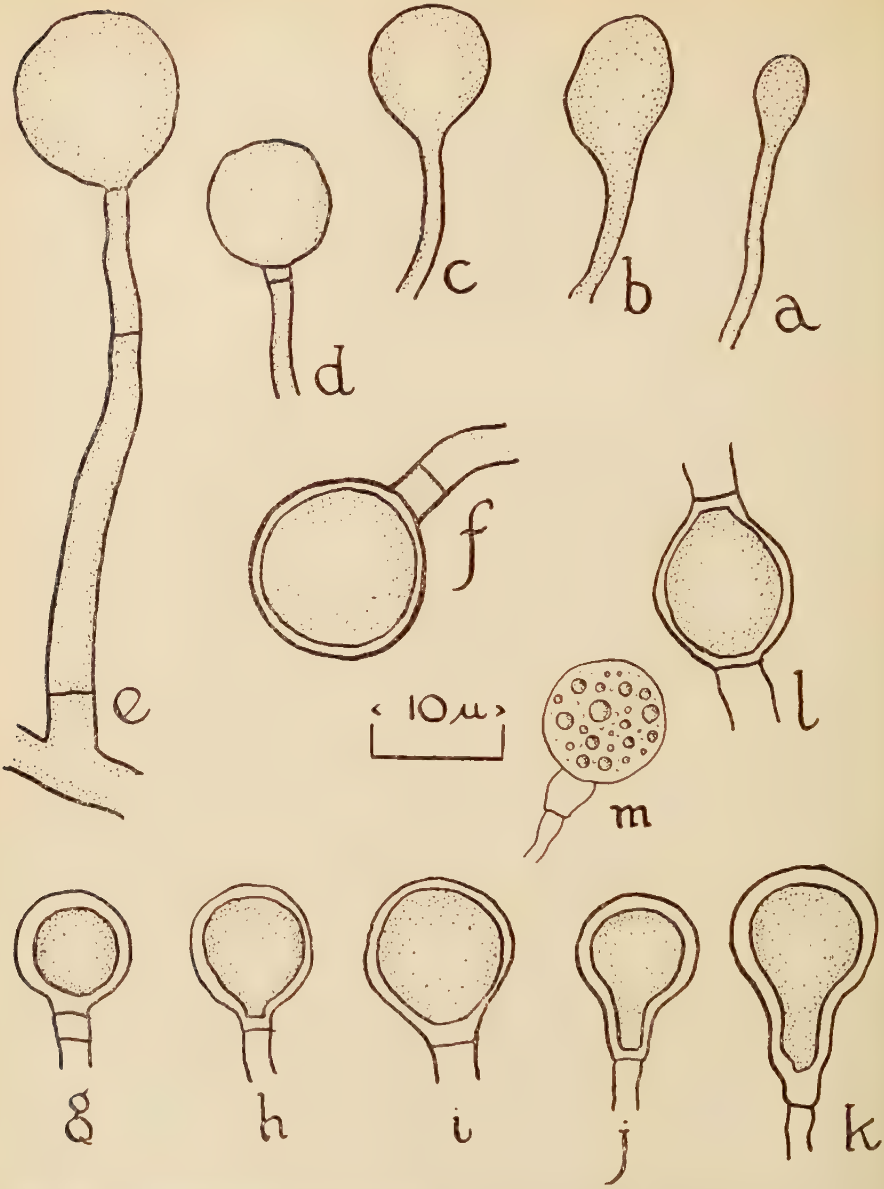
The conditions influencing the formation of thallospores are not clear. Temperature and pH variations which adversely affect growth had no effect on their occurrence. Colonies grown at low humidities, e.g. 76 per cent. R.H., developed very slowly, ascus formation being delayed for some weeks, but while thallospores were always present in such cultures, they were in no special abundance.



Text-fig. 6. *E. albus*: variation in spore number per ascus.

The method of dissemination of the thallospores and their exact function in the life-history of the fungus are also uncertain. In old slide cultures the hyphae just below the thallospores dissolve away leaving them free. Attempts to germinate thallospores isolated by micromanipulation were unsuccessful. The thick wall of the thallospore suggests a resting function, perhaps tiding the species through severe conditions; however, the ascospores themselves are still viable after 3 years, the age of the oldest spores available for test.

Eidam (1883, his Fig. 24) recorded a parthenogenetic ascus borne at the end of a hypha, and it was thought possible that the thallospores in the present isolates might be such structures. No delimitation of ascospores was ever observed, however, and they are not considered to be concerned with ascospore formation in any way. Eidam's Fig. 25 (reproduced here as Text-fig. 7, *m*) bears a close resemblance to the thallospores in the present material (Pl. III, *e*). This structure, regarded by Eidam as an abortive parthenogenetic ascus, might conceivably have been a thallospore comparable to those found



TEXT-FIG. 7. *E. albus*: thallospore formation. (*m* is after Eidam, 1883, and is not on the same scale as the other figures. It is approximately 18 μ in diameter.)

in the new isolates. The absence of a thickened wall can be accounted for by the impermanence of his material. The thallospores in the present isolates of *E. albus* have an average diameter of about 10 μ with a range of 7–17 μ. The structure represented in Eidam's Fig. 25 has a diameter of about 18 μ, so that the size is comparable.

f. Ascospore formation in the hyphae of Eremascus albus

Eidam (1883) recorded rare instances of abnormal ascospore formation, and figured ascospores in an ascus formed without the production of gametangia and situated at the end of a row of enlarged hyphal cells (his Fig. 24). Such asci were spherical and of normal size. Similar terminal asci were described by Stoppel (1907), but she decided that such structures in *E. fertilis* were not parthenogenetic in origin but arose from a normal gametangial copulation in which observation of the gametangia was difficult. She did, however, record parthenogenetic ascus development and development of ascospores in swollen hyphal cells, which was also observed by Guilliermond (1909).

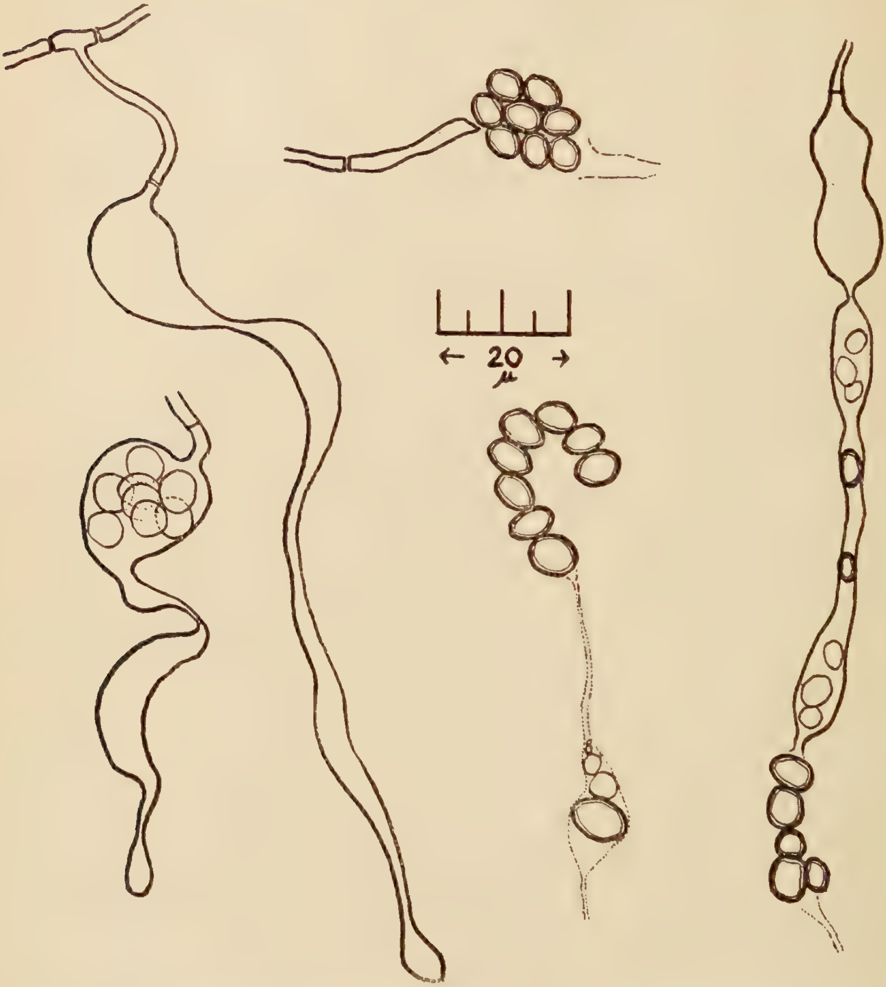
In older cultures of the present isolates of *E. albus* ascospores were formed in enlarged cells of some hyphae. Eidam's account makes no mention of such a process, although Fig. 4 in his paper shows hyphae with numerous intercalary and terminal swellings which may correspond to those associated with abnormal ascospore formation in the present material. There is wide variation in the number and grouping of the ascospores during such anomalous development (Text-fig. 8; Pl. III, *g*); the ascospores may be delimited in a single row or grouped together as in normal asci. In many instances irregular swelling of the hyphae without septa-formation takes place. When less than eight spores are present, small abortive spores can often be seen alongside the others which may or may not be greatly enlarged.

It has not been possible to obtain adequately stained preparations of hyphae undergoing abnormal ascospore development; and since no details of nuclear behaviour during this process are known, it is impossible to decide whether such development is due to true parthenogenesis or the fusion of two nuclei inside a hyphal cell. The ascospores in the hyphae are often in groups of eight, and this is regarded as presumptive evidence for supposing that a nuclear fusion has taken place in the hyphal cells followed by meiosis and then mitosis as in normal ascus development.

This situation in *E. albus* is closely paralleled in *E. fertilis*; Guilliermond's Fig. 42 (1909) shows a hypha with a series of aseptate swellings containing ascospores in varying numbers, closely resembling those in the new isolates of *E. albus*. In *E. fertilis* the gametangia commonly do not fuse, or may arise singly, and swell up into parthenogenetic asci containing 4 to 8 ascospores. Development of asci from single gametangia or from gametangia which have not fused was not observed in the present cultures of *E. albus* nor was such development described by Eidam (1883). No terminal asci corresponding to those found by Eidam were found in the new isolates. Beyond the fact that it is only in old cultures (upwards of two months old) that ascospores are produced in the hyphae, nothing is known of the exact conditions influencing such development.

Biggs (1937) describes the development of abnormally small intercalary asci in cultures of *Dipodascus uninucleatus* grown on a 1 per cent. lactose agar.

She recorded 'undifferentiated hyphal cells which may contain less than eight spores', and noted that 'in some cases there is evidence of a previous fusion of cells in the formation of these asci, but the smaller ones may be developed parthenogenetically'; her figures bear a striking resemblance to the comparable situation in *E. albus*. *Dipodascus*, *Eremascus*, and *Endomyces*, therefore, all exhibit ascospore formation unassociated with gametangial fusion, so that the phenomenon is apparently widespread in the Endomycetales.



TEXT-FIG. 8. *E. albus*: ascospore formation in hyphae. (Dotted lines represent the hyphae disintegrating to liberate the ascospores.)

IV. TAXONOMIC CHARACTERS OF THE SPECIES OF *EREMASCUS*

Cultures of the new isolates ascribed to *E. albus* were compared with cultures of *E. fertilis* during the course of identification. Three cultures of *E. fertilis* from the following sources were obtained for this purpose:

A: from honeycomb; isolated by Maurizio, 1939.
B: from shortcake; isolated by Stoppel, 1941.
(A and B came from the Centraalbureau voor Schimmelcultures, Baarn.)
C: from plum jam; isolated by the writer in Cambridge, 1948.

Several new facts concerning the cultural characters and ascospore size in both species came to light during the comparison which confirmed the original identification of the new isolates on the basis of gametangial characters. Thus, coiling of the gametangia in *E. albus* was frequent, but rarely occurred in *E. fertilis*. The almost spherical ascospores of the new isolates, apart from C above, also resembled the ascospore shape of Eidam's species rather than the smaller elliptical spores of *E. fertilis*.

The specific differences between *E. albus* and *E. fertilis* according to the original accounts (Eidam, 1883; Stoppel, 1907) are:

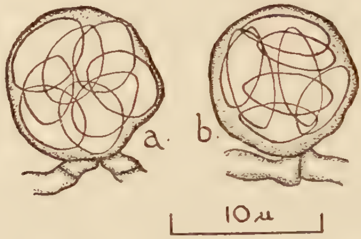
	Ascospore size.	No. of gametangial coils.
E. albus .	$5{\cdot}2-5{\cdot}5\,\mu$	1–4
E. fertilis .	$5{\cdot}2\times 3\,\mu$	1, rarely more, often none

E. albus also produces groups of asci on the same part of a hypha, although each ascus is developed on an independent pair of gametangia. Stoppel (1907) and Guilliermond (1909) do not show more than two asci arising on the same part of a hypha, and this is confirmed in the present isolates of this species.

The malt-sucrose agars used to maintain the present isolates of *E. albus* and *E. fertilis* represent a considerable advance on the media used by Eidam and Stoppel. *E. albus* will not grow on malt agar alone unless the humidity is reduced to about 98 per cent. R.H. or below, and *E. fertilis* produces only a slow-growing corrugated colony on this medium (Pl. IV, *i*, *a*). Both moulds produce a normal convex colony when grown on a malt-sucrose agar; they have well-developed hyphae, and they have retained their characters through numerous sub-cultures during 3 years. The following characters appear to be constant in the two species. (The standards used in describing colony colour are those found in the Horticultural Colour Chart issued by the British Colour Council in collaboration with the Royal Horticultural Society. Equivalent colours on the Ridgway Scale are given where possible.)

Eremascus albus—colony characters. On malt–40 per cent. sucrose agar a pure white colony is formed; at first convex but later becoming concave as the aerial mycelium dies down. Finally, the entire colony surface is covered with a mass of liberated ascospores and assumes a powdery texture (Pl. III, *h*). The same characters are found in colonies on malt–40 per cent. sucrose–0·5 per cent. Difco yeast agar, but the growth rate and sporulation are increased; as the aerial mycelium dies down the colonies become yellowish. This colour was present in colonies growing naturally on mincemeat and jam substrates (isolates 4 and 5) and is a Primrose Yellow (60. 1/3 = Pale Lemon Yellow, 23. B Ridgway).

Ascospore size. The ascospores of *E. albus* are almost spherical while those in *E. fertilis* are elliptical with pointed ends. Strain B of *E. fertilis* showed wide variation in ascospore shape, some of the spores resembling those of *E. albus* rather than those of typical *E. fertilis* (Text-fig. 9). Ascospore measurements of both species were made from the original material where available and from 3-week cultures on different media at 25° C. From each culture samples of 200 spores (mounted in water) were measured; details of the average measurements are given below in Tables I and II.



TEXT-FIG. 9. *E. fertilis*: culture B, showing wide variation in ascospore shape in asci on the same mycelium.

TABLE I

Average Ascospore Size in Eremascus albus

Isolate.	Substrate.	Ascospore size (µ).
1	Mustard powder	7·5×6·3
	Dried M (at 90% R.H.)	7·5×6·3
1	M 40	7·5×6·3
	M 40 Y	7·5×6·3
2	M 40	8·1×6·9
	M 40 Y	7·5×6·9
4	Mincemeat	7·5×6·9
	M 40 Y	8·1×6·3
5	Jam	8·1×6·9
	M 40 Y	7·5×6·9
6	M 40 Y	7·5×6·3

M = 2% malt extract agar; M 40 = 2% malt–40% sucrose agar; M 40 Y = 2% malt–40% sucrose–0·5% Difco yeast agar.

The average spore dimensions of *E. albus* are 7·7×6·5 µ, with the size 7·5×6·3 µ as the most frequently occurring class. The range lies between 3·8–11·5 µ in length and 3·1–10·0 µ in breadth, these extremes being accounted for by the occurrence of single large and small ascospores in asci; no asci contain all large or all small spores of the extreme dimensions. The present estimate of ascospore size in *E. albus* differs considerably from Eidam's original measurements (5·2–5·5 μ), but no further comparison is possible since Eidam's material did not survive. It is proposed that the present estimate be taken as the ascospore size of *E. albus* since there is good agreement between the separate estimates for the five new isolates of this species.

Size of asci, thallospores, and hyphae. Eidam (1883) gave the diameter of the asci of *E. albus* as 12·5–13 μ, but in the present isolates, where the ascospores are larger, the ascus diameter is 15 μ (range 12–19 μ), measured when the ascospores are fully formed (i.e. with a double wall) but while the ascus wall is still intact.

Thallospore size in the present material cannot be compared with any corresponding estimate in the original description. The average diameter of thallospores in *E. albus* is 10 μ (range 7–17 μ) with 7, 8, and 9 μ as most frequent.

The hyphae in *E. albus* are 3–4 μ in diameter.

The following diagnosis based on the characters of the five new isolates is proposed:

Eremascus albus: Colony regular, convex; white, ageing to primrose yellow; hyphae septate 3–4 μ diameter. Thallospores with thickened walls, 10 μ diameter, terminal or intercalary. Asci spherical, 15 μ diameter, naked and solitary on pairs of coiled gametangia; 1–4 gametangial coils; several gametangia may arise on the same part of a hypha. Ascospores almost spherical, 7·7 × 6·5 μ; eight per ascus, liberated by dissolution of the ascus wall.

Eremascus fertilis. Stoppel (1907) laid no special emphasis on the cultural characters of her isolate, recording only that a 15 per cent. apple-jelly medium gave the best growth. The present cultures (A, B, and C) on 2 per cent. malt agar produce a slow-growing corrugated colony. Lateral spread is very slow and the hyphae are composed of irregular cells. Pl. IV, *i*, *a* shows a colony at 25° C. on malt agar, 2 months old. On malt–40 per cent. sucrose agar a flat white colony with sparse aerial mycelium develops (Pl. IV, *i*, *c*), which is quite different from the dense corrugated colony produced on malt–40 per cent. sucrose–0·5 per cent. Difco yeast agar (Pl. IV, *i*, *b*). Such colonies are white at first but gradually change to amber yellow (50. 5/3; no Ridgway equivalent). In colonies on the sucrose agars the hyphae are typical and contrast sharply with the short distorted hyphae produced on malt agar.

TABLE II

Average Ascospore Size in Eremascus fertilis

Culture.	Substrate.	Ascospore size (μ).
A	M 40	6·3 × 3·8
B	M 40	6·9 × 3·8
	M	5·6 × 3·1
C	Jam	6·3 × 3·1
	M 40	5·6 × 3·1
	M	5·6 × 3·8

(For explanation of M and M 40 see Table I.)

Ascospore size. The average dimensions are 6·1 × 3·5 μ, with 6·3 × 3·8 μ as the most frequent class. The range lies between 3·8–10·0 μ in length and 2·5–5·0 μ in breadth. The ascospores of the present cultures are larger than those of the type species (Stoppel, 1907, 5·2 × 3·0 μ), but the discrepancy is small and no useful purpose would be served by designating these cultures as different varieties, since comparison with the original ones is impossible.

V. CYTOLOGICAL STUDIES

No cytological studies were carried out on *E. albus* by Eidam (1883). Stoppel (1907) and Guilliermond (1909) investigated the cytology of *E. fertilis*, but they were unable to make out chromosomes in their material.

Materials and methods. Germ-tube material was obtained by adapting a cellophane strip technique used by Robinow (1942) in his studies of bacterial cytology. Strips of permeable cellophane (British Cellophane Company, No. 300) were sterilized by autoclaving in a tube of water and then placed on malt-sucrose agar in a Petri dish after removing the excess of water between sheets of sterile blotting-paper. Ascospores were dusted on to the cellophane surface and then incubated. The strip bearing the germ-tubes was then lifted off and placed in the fixative and stained. Material of the asci was obtained in essentially the same way, incubated at 25° C. over a 16 per cent. saline solution until suitable stages of ascus formation developed. This required 6 days for *E. albus* and 7 days for *E. fertilis*. Development at 90 per cent. R.H. (approximately the value over 16 per cent. saline solution in a Petri dish) is faster than in a dish of malt–40 per cent. sucrose agar where the relative humidity is about 98 per cent. Relative humidity is a very important factor controlling the growth of *E. albus* and will be discussed in detail in a second paper. Use of the method outlined above saves considerable time in producing asci for cytological studies.

Acetocarmine and similar stain-fixatives applied with or without prior fixation of germ-tubes in acetic alcohol (1 : 3) were not successful. Aqueous fixation with La Cour's 2BE fluid was satisfactory, and if the germ-tubes were less than 4 days old they did not float off from the cellophane strip during fixation and washing. Fixed germ-tubes were stained with iron alum-haematoxylin according to the schedule of Darlington and La Cour (1942). This technique revealed the nuclei as light circular areas each with a deeply staining nucleolus. No details of chromosomes or spindles were visible, and variations in times of fixation, mordanting, and differentiation gave no improvement.

The Feulgen technique was applied to germ-tubes and asci without success.

Treatment overnight in acetic alcohol gave good fixation of the asci, but since acetic alcohol is not a satisfactory fixative for subsequent haematoxylin treatment, material fixed in this way was mordanted in a chromic acid solution (e.g. La Cour's 2BE fluid without the addition of osmic acid) for 30 minutes, followed by thorough washing prior to staining.

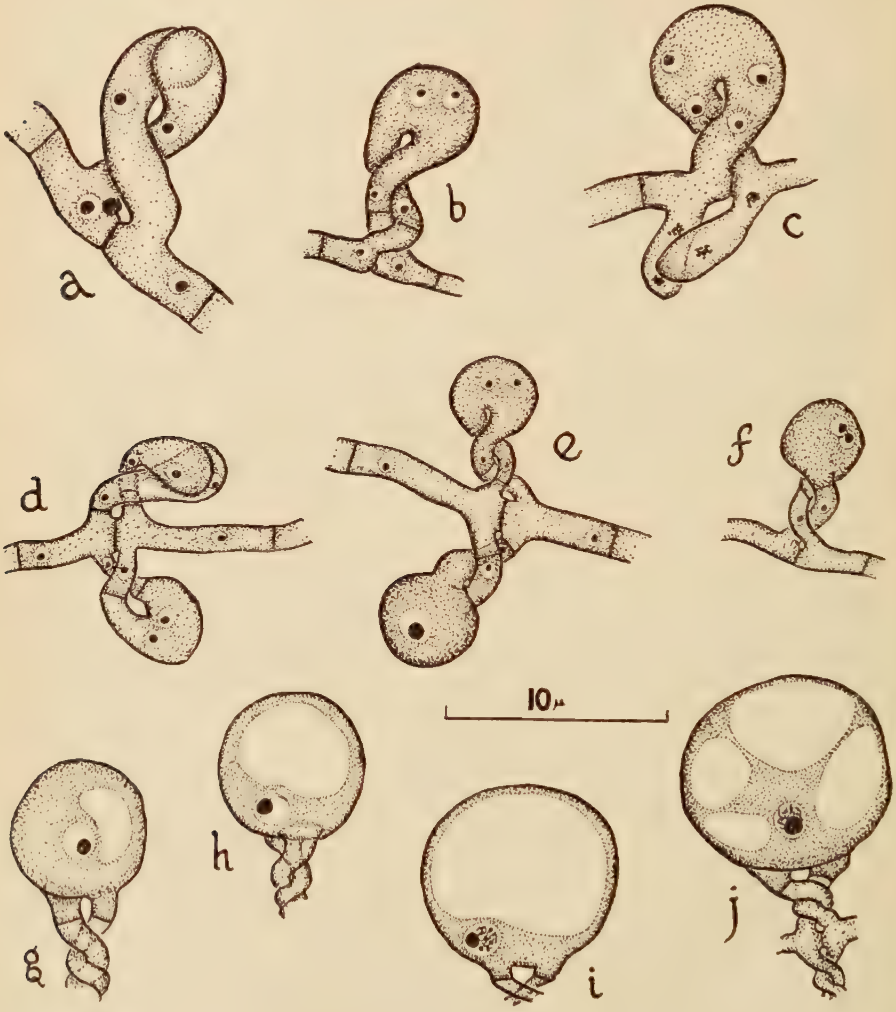
Haematoxylin preparations gave a good picture of resting nuclei during ascus formation, but details of chromosomes during nuclear division were obscure. The modified acetocarmine squash method described below gave much clearer pictures of the chromosomes but left the resting nuclei unstained. Each method, therefore, was used to supplement the other in studying the nuclei during ascus development.

Studies of the chromosomes of *Neurospora crassa* (McClintock, 1945) and *N. tetrasperma* (Cutter, 1946 *a*, *b*), using the acetocarmine method without modification, have lately appeared. Material of *E. albus* was subjected to similar treatment without success. Fincham (1949) working with *Neurospora crassa* and *N. sitophila* likewise found that the methods of McClintock and Cutter did not suit his material unless the following modification, suggested by Godward (1948) for algae, was used. Material of *E. albus* (fixed in acetic alcohol) was mordanted in 4 per cent. iron alum solution for periods of 30 seconds, 1, 3, 5, 10, and 30 minutes, and subjected to the usual acetocarmine squash method after washing out the excess iron alum. This technique gave satisfactory chromosome preparations, the best results being obtained after 5 minutes' mordanting or less. Mordanting for 10 minutes or more caused too deep staining of the cytoplasm. In *E. albus* only the dividing nuclei are properly stained; the resting nucleus is poorly differentiated, a nucleolus is rarely distinguishable, and no details of the prophase of mitosis or meiosis can be seen.

Results

Eremascus albus. The ascospores are uninucleate when germination begins; as the spore pushes out the vesicle from which the germ-tubes arise the nucleus divides. Further divisions follow rapidly and as the germ-tubes grow the spore case becomes empty. The apices of the germ-tubes are multinucleate; as many as twenty nuclei were counted in one long terminal cell. After 3–4 days the germ-tubes have developed into segmented hyphae with uninucleate cells, which is the predominant adult condition, although multinucleate cells can be found occasionally. Division figures in the germ-tubes and hyphae were too small for chromosome counts to be made.

The gametangia arise from adjacent pairs of uninucleate cells. As the parent cell forms the outgrowth which will become the gametangium its nucleus divides, and one of the daughter nuclei passes into the outgrowth leaving the other within the parent cell (Text-fig. 10, *a*, *b*). A cross-wall is laid down (Text-fig. 10, *b*), and the parent cell may then give rise, by a similar process, to further gametangia close to the first (Text-fig. 10, *c*, *d*, *e*). Once the gametangium is cut off from the parent cell (Text-fig. 10, *b*), or before this happens (Text-fig. 10, *c*), its nucleus divides once and the upper one passes to the tip of the gametangium where it is destined to act as a gamete. This division takes place prior to fusion of the gametangia (Text-fig. 10, *d*); after this fusion has taken place the gamete nuclei (which have no special characters making them visibly different from each other or other nuclei) meet and fuse (Text-fig. 10, *e*, *f*). There is no synchronization between the development of a number of gametangial pairs from the same parent cells. Thus Text-fig. 10, *e* shows fusion of the lower gametangia whilst the two gamete nuclei are still approaching each other in the upper pair. The gamete nuclei and the fusion nucleus arising from them may be cut off from the lower part of the gametangium before or after fusion has occurred.



TEXT-FIG. 10. *E. albus*: nuclear behaviour during ascus formation. (Iron-alum haematoxylin stain.)

Swelling of the fused tips of the gametangia to form the ascus takes place after the nuclear fusion; such swelling is correlated with the appearance of a vacuole alongside the fusion nucleus which is generally at the base of the ascus and to one side (Text-fig. 10, *g*, *h*, *i*). This vacuole disappears before the ascospores are cut out (Text-fig. 10, *j*). During vacuolation and devacuolation the fusion nucleus passes into the prophase of the ensuing meiosis (Text-fig. 10, *i*, *j*). The nucleus undergoes three divisions (Pl. IV, *k*, *l*, *m*) and since only one nuclear fusion occurs there can be no question of brachymeiosis. The ascospores differentiate around the eight nuclei and are formed in the manner typical of the Ascomycetes.

Great effort was made to analyse the details of the nuclear divisions in the ascus, but no completely satisfactory preparations were obtained from which unassailable information concerning chromosome number and structure could be derived. Pl. IV, *j* shows metaphase I during meiosis.

Eremascus fertilis. The situation was more difficult in *E. fertilis* where the asci and nuclei are even smaller than in *E. albus*. Stoppel (1907) and Guilliermond (1909) had no success in seeing chromosomes in their material. Using the technique described above, acetocarmine preparations of the chromosomes during ascus division were obtained, but it proved impossible to count the chromosomes in this species as they were too small.

VI. Discussion

A normal process of isogamous fusion leading to ascus-formation in *E. albus* has been described, together with variations towards anisogamy and fluctuations in ascospore number per ascus. These facts may be adduced to support a derivation of the Ascomycetes from the Phycomycetes in which the Endomycetaceae and forms like *Ascoidea* and *Dipodascus* resemble transition types between the two groups. Such a derivation has been postulated by many mycologists, notably de Bary (1884), Atkinson (1915), and Gäumann and Dodge (1928), who stated that 'the step from the Zygomycetes to the Hemiasci (i.e. Hemiascomycetes) would consist in the differentiation of the germ sporangium to an ascus conditioned by the privileging of sexual nuclei, while the structure of the sexual organs themselves and the place of caryogamy remains unaltered'.

Ascoidea rubescens (Walker, 1931, 1935) is a form showing both phycomycete and ascomycete characters and is probably a specialized line away from the main path of evolution, since Walker could find no evidence of sexual fusion at any stage of the life-history. The spore sac of *A. rubescens* is therefore best regarded as an apogamously developed gametangium. *Dipodascus albidus* (Lagerheim, 1892; Juel, 1902, 1921) is closer to the suggested evolutionary line, and has the phycomycete character of a multispored 'sporangium' together with the predominantly ascomycete characters of free cell-formation of the spores and direct development of the fused gametangia to produce the 'ascus'. Biggs (1937) reported another species, *D. uninucleatus*, in which the cells of the hyphae and gametangium are uninucleate, bringing this species indubitably into the ascomycete group and indicating the form of the probable transition types between *D. albidus* and members of the Endomycetaceae.

The line of evolution suggested above requires a reduction in the spore number in ancestral multispored forms resembling *D. albidus*, culminating in the eight or four spores per ascus of *Eremascus* and *Endomyces*. Atkinson (1915) remarked that in older cultures of *D. albidus* the asci became smaller and the spores less numerous, suggesting a tendency towards the reduced number in *Eremascus*. We have seen that *E. albus* (and also *E. fertilis*) can produce more than eight spores per ascus (Text-fig. 6, *b*) indicating that this character is not fixed; the possibility of some ancestral spore number greater than eight is thus not precluded. Morphological resemblances between the sexual processes in *Dipodascus albidus* and *E. albus* have also been noted, which support the idea of relationship between *Dipodascus* and members of the Endomycetaceae. The variations in the gametangia of *E. albus* also indicate a way in which the antheridia and ascogonia of higher Ascomycetes may have evolved from an isogamous ancestry.

The Endomycetales comprise very simple forms, and it is necessary to decide whether this simplicity is primitive or due to reduction caused by the specialized habitats in which many of these fungi live; thus, the Saccharomycetaceae are generally accepted as reduced forms specialized for living in sugary habitats. *E. albus* cannot grow in saturated conditions and its habitat range is therefore limited in comparison with most fungi. None of the Endomycetaceae show much development of conidia or special mechanisms for ascospore dispersal, and their ascospores are often resistant and long-lived. Thus, ascospores of *E. albus* will germinate successfully 3 years after their formation, and *Endomyces fibuliger* produces mycelial buds which can survive long periods at 55° C. (Gäumann and Dodge, 1928, p. 141). These moulds stand in sharp contrast to the ubiquitous Aspergilli and Penicillia with their highly developed methods of spore production. The Endomycetales comprise many forms living in special habitats of spasmodic occurrence (e.g. slime fluxes) and have no well-developed method of spore dispersal. The spores themselves for the most part result from a sexual process or some modification of this; there is little development of asexual spores.

The members of the Endomycetaceae are homothallic and the tendency towards parthenogenesis is probably connected with the loss in significance of the sexual process through the fixed occurrence of homothallism. Heterothallism appears to be a necessary condition for the occurrence of progressive evolution in certain groups of Fungi, and perhaps it is possible to correlate the prevalent homothallism of the Endomycetaceae with an adaptation to special habitats where outbreeding mechanisms might not be helpful for continued survival. Alternatively, the occurrence of homothallism may have led to fixity of habitat.

The Endomycetaceae are therefore regarded as simple types in which further simplification due to reduction is found, e.g. the breakdown of the sexual process leading to parthenogenesis. Some, particularly *Eremascus*, still retain primitive characters such as a sexual process involving fusion of equal gametangia with direct development into a single naked ascus. *Eremascus* is regarded as closely resembling transitional forms on the suggested line of development of the Ascomycetes from the advanced Phycomycetes (e.g. *Endogone*) through intermediate stages indicated by the Hemiascomycetes (e.g. *Dipodascus*).

VII. SUMMARY

1. The Ascomycete *Eremascus albus* Eidam, lost since 1883, has been found again. Five new isolates from mustard powder, mincemeat, and jam have been obtained.

2. The original description has been generally confirmed and amplified.

3. The species is homothallic. Variations of the normal sexual process, ascus shape, and ascospore number are recorded and discussed.

4. The formation of ascospores inside enlarged hyphal cells and the occurrence of thallospores are described for the first time.

5. The taxonomic characters of the genus *Eremascus* are discussed, and cultural characters distinguishing the two species are described.

6. Ascospore size in the present isolates of *E. albus* is $7{\cdot}7 \times 6{\cdot}5\,\mu$; this differs appreciably from the size of the ascospores in Eidam's original description.

7. Ascospore size in *E. fertilis* is $6{\cdot}1 \times 3{\cdot}5\,\mu$; this differs slightly from Stoppel's original figure.

8. The cytology of *E. albus* during germination, gametangium- and ascus-formation is described for the first time, but the chromosome number could not be definitely determined.

9. The status of the Endomycetales is discussed.

VIII. ACKNOWLEDGEMENTS

This study was carried out during the writer's tenure of the Soley Studentship at King's College, Cambridge, and a maintenance grant from the Department of Scientific and Industrial Research. The writer wishes to thank Mr. F. T. N. Elborn for most of the photographs. I am indebted to Professor F. T. Brooks, who first reisolated the lost species and handed it to me, for advice and encouragement throughout the investigation.

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EXPLANATION OF PLATES

Illustrating C. E. Harrold's article 'Studies in the Genus *Eremascus*, I. The Rediscovery of *Eremascus albus* Eidam and Some New Observations concerning its Life-history and Cytology.'

j, *k*, *l*, *m*: iron-alum acetocarmine squash preparations. *a–f*, ×700; *g*, ×500; *h*, *i*, ×1; *j–m*, ×2,500.

a. *E. albus*: Germination of ascospores. Note small vesicle out of which the germ-tubes grow.

b. *E. albus*: Gametangia coiling.

c. *E. albus*: Fusion of coiled gametangia.

d. *E. albus*: Fusion of gametangia, swelling of ascus, and delimitation of ascospores.

e. *E. albus*: Terminal thallospores.

f. *E. albus*: Thallospores on germ-tubes.

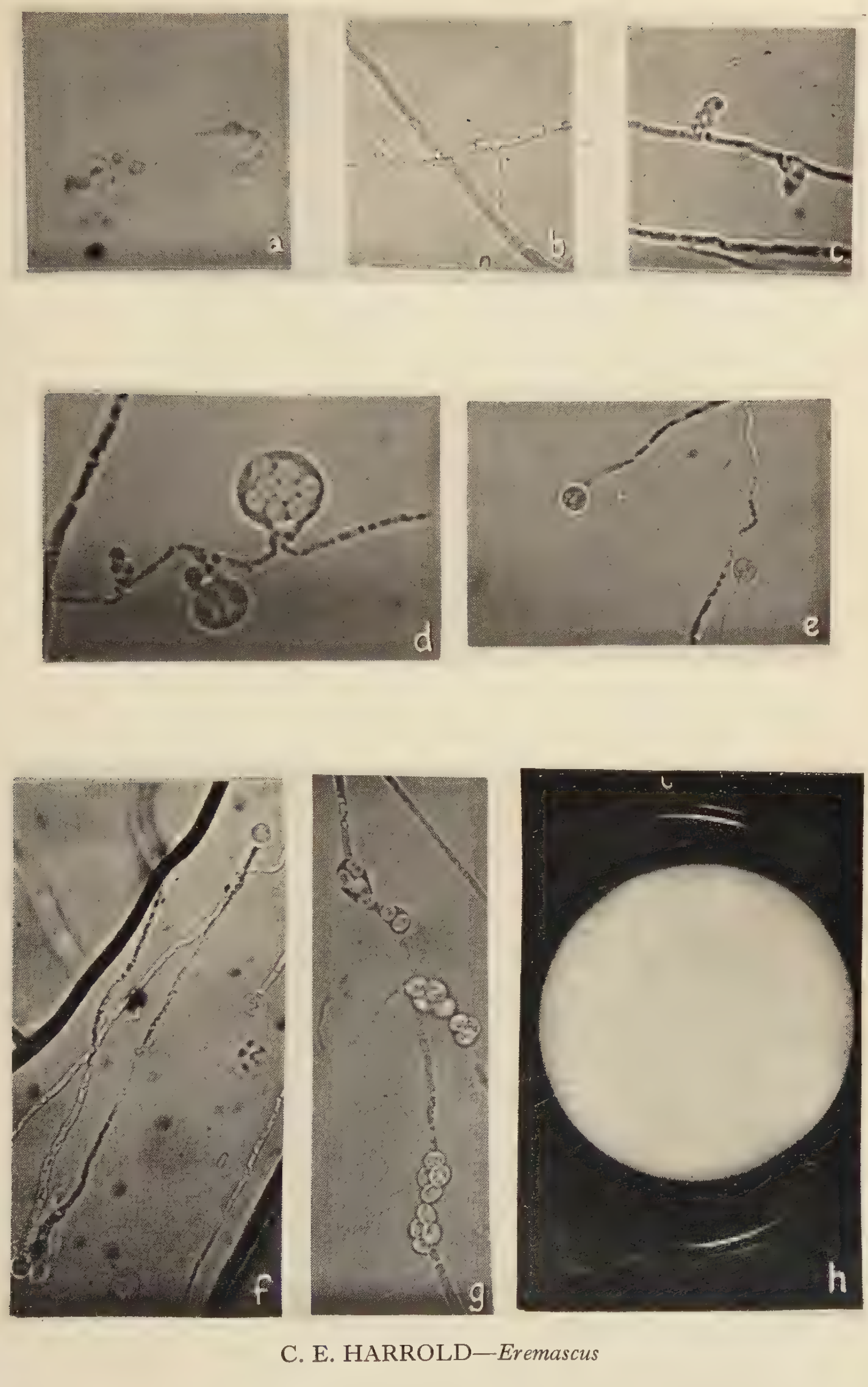
g. *E. albus*: Ascospore development in hyphae.

h. *E. albus*: Colony on malt–40% sucrose–0·5% Difco yeast agar.

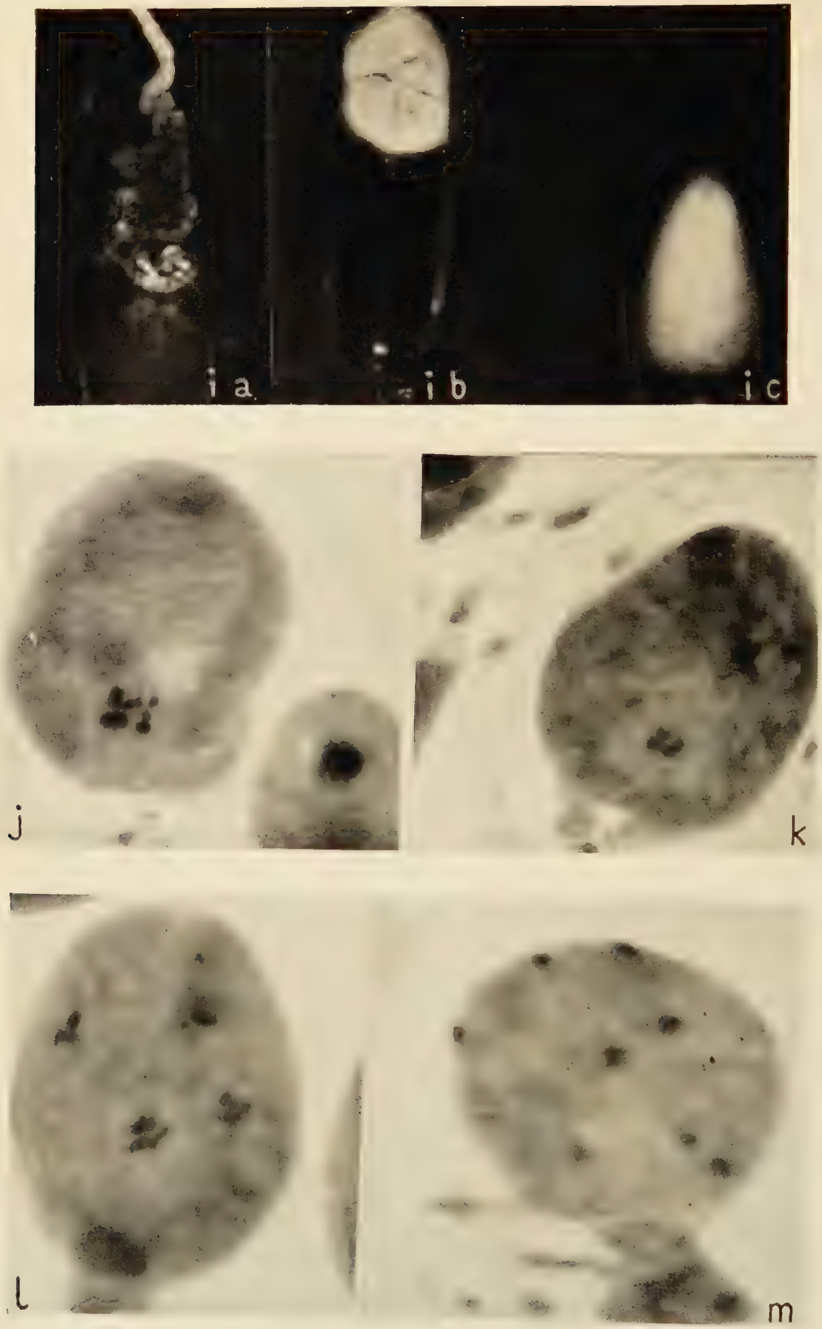
i. *E. fertilis*: Colonies on (*a*): malt agar; (*b*): malt–40% sucrose–0·5% Difco yeast agar; (*c*): malt–40% sucrose agar.

j. *E. albus*: Metaphase I of meiosis in the ascus.

k, *l*, *m*. *E. albus*: Nuclear divisions in the ascus showing successive formation of 2, 4, and 8 nuclei.



C. E. HARROLD—*Eremascus*



C. E. HARROLD—*Eremascus*

Wilt of Cacao Fruits (*Theobroma Cacao*)

V. Seasonal Variation in Potassium, Nitrogen, Phosphorus, and Calcium of the Bark and Wood of the Cacao Tree

BY

E. C. HUMPHRIES

(*Rothamsted Experimental Station, Harpenden, Herts.*)

With two Figures in the Text

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INTRODUCTION

IN the previous paper of this series (Humphries, 1947) the results of an experiment were described in which the seasonal changes in water content and carbohydrate reserves (starch, sucrose, reducing sugars, alcohol-soluble material) as well as non-carbohydrate alcohol-soluble material at three levels in the bark and wood of the cacao tree were followed during a period of 14 months. It was established that significant changes in most of these constituents occurred in response to vegetative changes and to crop production. No evidence was obtained that the available carbohydrate supply limited the size of the crop and it appeared probable that the supply of one or other of the mineral elements was the critical factor.

At the same time that the samples for carbohydrate estimations were taken, subsamples were oven-dried for subsequent mineral analysis. It is the purpose of the present paper to discuss the seasonal changes in certain elements and at the same time to see how these are related to the carbohydrate changes. The data have been subjected to the same scheme of statistical analysis as in the previous paper to establish whether significant changes between seasons and levels occur.

SEASONAL CHANGES IN THE WOOD

The mean values of the mineral elements present in the wood are shown in Table I together with the degree of significance between seasons and between

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levels. There is a highly significant seasonal variation in *potassium*. The difference between levels is also significant, and as the greatest amount occurs at the 1-ft. level there is a negative gradient of this element in the wood. The seasonal changes of potassium in the wood are positively correlated with changes of this element in the bark. The potassium figures for wood and bark are shown in Appendix, Table I. In the case of *phosphorus* there is also a highly significant difference between seasons and between levels and a consistently negative gradient (see Appendix, Table IV). Total *nitrogen* shows no significant seasonal variation and no significant difference between levels (Appendix, Table III). There is no seasonal change in *calcium*, but a negative gradient exists between the levels investigated.

Table I

Mean Content of Mineral Constituents in Bark and Wood (g. per 100 g. Water), Degree of Significance of Difference between Levels and Seasons, and Correlation between Like Constituents of Bark and Wood

	BARK.			WOOD.					
		Degree of significance (*P*) of difference between			Degree of significance (*P*) of difference between		Correlation between bark and wood.	Direction of gradient.	
	Mean.	Levels.	Seasons.	Mean.	Levels.	Seasons.	Seasons.	Bark.	Wood.
Potassium	0·438 ± 0·009	0·05	0·001	0·453 ± 0·005	0·001	0·001	+0·755 (*P* = 0·01)	−ve 1 ft. to 6 ft. +ve 6 ft. to 12 ft.	−ve
Calcium	3·688 ± 0·060	0·001	0·05	0·894 ± 0·021	0·01	Not sig.	+0·338 not sig.	−ve	−ve
Phosphorus	0·031 ± 0·0004	Not sig.	0·05	0·047 ± 0·0019	0·001	0·01	+0·509 not sig.	0	−ve
Nitrogen	0·382 ± 0·004	0·01	0·05	0·372 ± 0·008	Not sig.	Not sig.	+0·471 not sig.	−ve 1 ft. to 6 ft. +ve 6 ft. to 12 ft.	0

The data were examined to see if they revealed any marked trend during the dry season and especially whether there was any indication of restricted uptake during this period. It may be inquired what conditions, according to current conceptions of mineral transport, would exist in the tree if the uptake of minerals by the roots is reduced during the dry season. It is generally accepted that the wood is the upward channel of transport of mineral substances and assuming that the rate of uptake from the soil is fairly constant, as might be expected under the fairly uniform soil and atmospheric environments which exist during the wet season in Trinidad, the total gradient in the wood of any particular element should be reasonably constant (assuming that the static or structural gradient of this element is also constant). This assumption is supported in the present case by the data in Fig. 1, where the gradients of calcium, potassium, phosphorus, and total nitrogen in the wood between the 1-ft. and 12-ft. levels are plotted. It will be noticed that there are negative gradients of calcium, potassium, and phosphorus, and moreover that these gradients do not vary much during the period June–December, that is, during the wet season. There is, however, a diminution in the gradient during the dry season, but the extent of this decrease varies with the different elements. In the case of calcium there is a steady decrease in gradient from the onset of the dry season (January) until the gradient has virtually disappeared after 5 months of dry weather. During the ensuing wet season the gradient quickly becomes established again. With potassium there is not such a marked change of gradient at the beginning of the dry season but it falls during the last month to a low value. There appears to be no change of the gradient of phosphorus during the whole period of the experiment and therefore no dry-season effect. The differences in gradient changes of these three elements seem best interpreted, not as an effect of drought on the differential uptake of these elements, but as connected with differences in redistribution

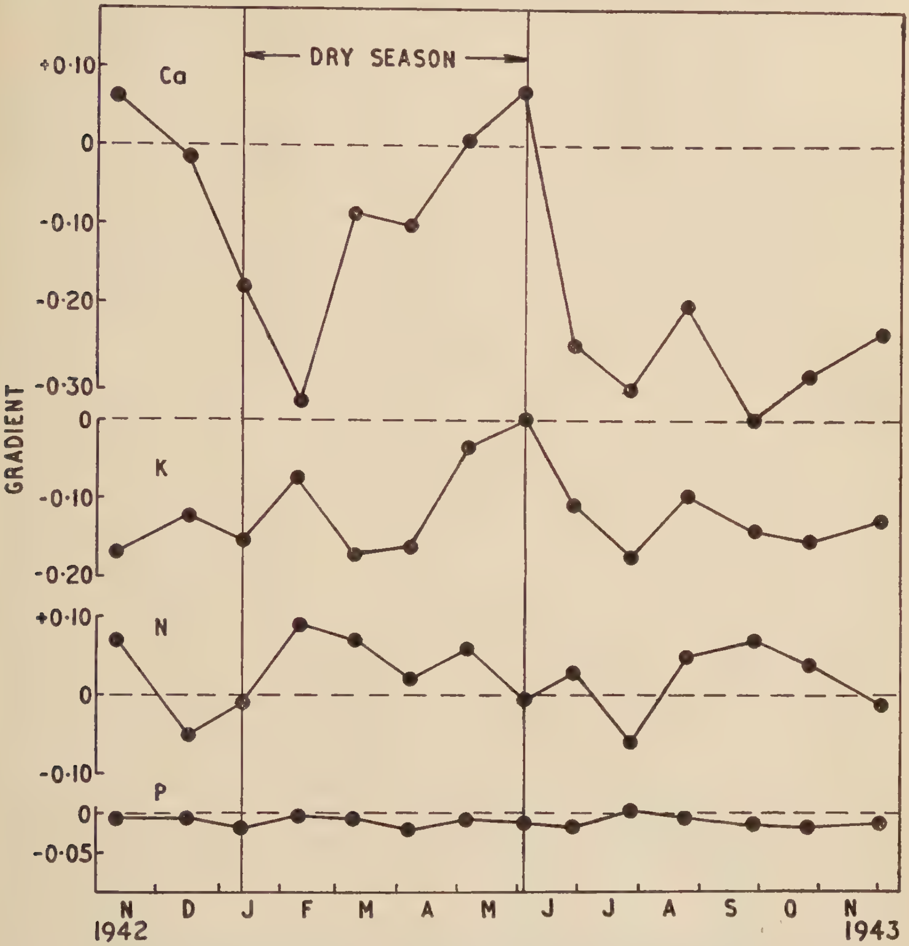

FIG. 1. Seasonal changes in gradients of calcium, potassium, total nitrogen, and phosphorus, g. per 100 g. water, in the wood between the 1-ft. and 12-ft. levels.

of the elements within the plants when the supply from the soil has been reduced to a low level. The work of Mason and Maskell has shown that calcium is not phloem-mobile and is not re-exported from tissues which it reaches via the transpiration stream. In other words, there is little possibility of redistribution of calcium, and hence this element would be expected to be the first to show a diminished gradient when uptake from the soil is restricted. In the case of potassium and phosphorus, however, Mason and Maskell have shown that in the cotton plant these elements are readily re-exported via the phloem and can leak back into the xylem vessels. If this occurs in the cacao tree in the dry season, a gradient of these elements could readily be maintained by transport from the bark into the wood at the same level even though there is no net uptake from the soil. That potassium does show a decrease in gradient at the end of the dry season is probably connected with the fact that potassium tended to be deficient in the experimental trees, as will be shown later from other evidence. Thus the observations appear to be in accord with the supposition that there is a progressively diminishing uptake from the soil during the dry season and that by the end of the dry season this uptake has almost if not entirely ceased.

It is extremely likely that as the dry season progresses the transpiration rate increases, but unfortunately there is no direct evidence on this point. In the dry season there are several environmental factors which would tend to increase the transpiration rate. The cacao-shade trees (*Erythrina* spp.) lose their leaves during this season so that insolation is greatly increased. The atmospheric humidity is low and the amount and velocity of the wind increases. There is invariably a big vegetative flush (and sometimes two) at the height of the dry season in response to the higher temperatures (Humphries, 1944 *a*). All these factors would tend to increase the transpiration rate in spite of the diminished uptake from the soil. The fall in water content of the wood during the dry season is probably accounted for by a combination of these two factors. In the foregoing argument only the total gradients have been considered and it is not known what fraction of the total amount of each element is actually present in the transpiration current. If, however, the calcium, for instance, of the transpiration current ceases to be renewed, a decrease in calcium gradient would be expected. If the calcium of the transpiration stream is only a small fraction of the total calcium, then the predominating gradient of structural and accumulated calcium would show little change in response to drought. Since there is a response with the gradients decreasing as the season progresses, it suggests that either the total calcium is made up of quite a large proportion of transpirational calcium or that there is a redistribution of calcium in the wood during the dry season. This latter alternative is in conflict with the generally accepted idea that calcium is not readily remobilized and hence the former alternative appears to be the case.

The data for the calcium gradients plotted in Fig. 1 have been examined statistically to see if the dry-season gradients are in fact significantly lower than those of the wet season. It is found that the average gradient over the whole of the dry season is not significantly different from the average wet-season gradient, but it is not to be expected that the full effect of drought on uptake from the soil will be immediately evident as the drying out of the soil is a gradual process.[1]

The average value of the gradient in the last three months of the dry season, viz. −0·01 g. Ca, is significantly lower than the average gradient during the remainder of the period under investigation, viz. −0·20 g. Ca. These gradients expressed as g. per 100 g. water possibly exaggerate the magnitude of the difference between seasons, since there is a progressive decrease in water content of the wood during the dry season (Humphries, 1947), so that the gradients calculated on a dry-weight basis have also been examined. These show a similar effect and the gradients in the last two months of the dry season are significantly lower than in the rest of the period.

SEASONAL CHANGES IN THE BARK

Potassium

The potassium content of the bark is of the same order of magnitude as that of the wood (Table I and Appendix, Table I), but slightly lower in the bark at the 1-ft. and 6-ft. levels and slightly higher at 12 ft. Seasonal changes of total potassium in the bark are significant ($P = 0{\cdot}001$) and the levels differences are also significant. It may be seen from the data in Appendix, Table I, that the concentration of potassium is usually highest at the 12-ft. level and lowest at the 6-ft. level. The seasonal drifts in potassium contents of the three levels are not strictly parallel to one another, so that differences in *gradients* between the levels occur, and it is proposed to discuss the data in terms of gradients rather than consider the seasonal changes in concentration at any particular level. As mentioned above, the concentration of potassium in the bark is usually highest at the 12-ft. level and lowest at the 6-ft. level, so that there is a negative gradient between 1 ft. and 6 ft. and a positive gradient between 6 ft. and 12 ft. This could be explained by assuming that there is a negative structural and storage gradient which is relatively much steeper between 1 ft. and 6 ft. than between 6 ft. and 12 ft. This appears reasonable since the 1-ft. region is ontogenetically older than the 12-ft. region. It is further assumed that the storage gradient in the lower region completely masks the positive dynamic gradient. Between the 6-ft. and 12-ft. levels, however, the gradient is positive and here the dynamic gradient predominates over the relatively small storage gradient. As a consequence it may be assumed that the seasonal changes in gradient in the upper region are in the main a reflection of the changes in the positive dynamic gradient, while the changes in gradient in the lower region are due to changes in the storage gradient.

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[1] Data on the change in soil moisture (0–12 in.) during the dry season were obtained for the same estate and soil type in a previous experiment (Humphries, 1944*b*). There was an almost linear fall in gross soil moisture from about 25 per cent. in January to 16 per cent. in May at the end of the dry season.

The month to month changes in the total potassium gradient in the two regions are plotted in Fig. 2.

It was postulated by Mason and Maskell (1931) that so long as the supply of an element is relatively abundant, the cells of the bark continue to store the surplus that is not required for immediate growth. A negative storage gradient would then exist in the bark. If the supply were restricted, there might be no appreciable storage and the vertical gradients in the bark might then be positive, representing the positive dynamic gradient with its transport head in the foliage region. The present data appear to bear out this contention. As will be shown later, certain of the experimental trees are lower (and probably deficient) in potassium. It would be expected that the trees more deficient in potassium would tend to have greater positive gradients between

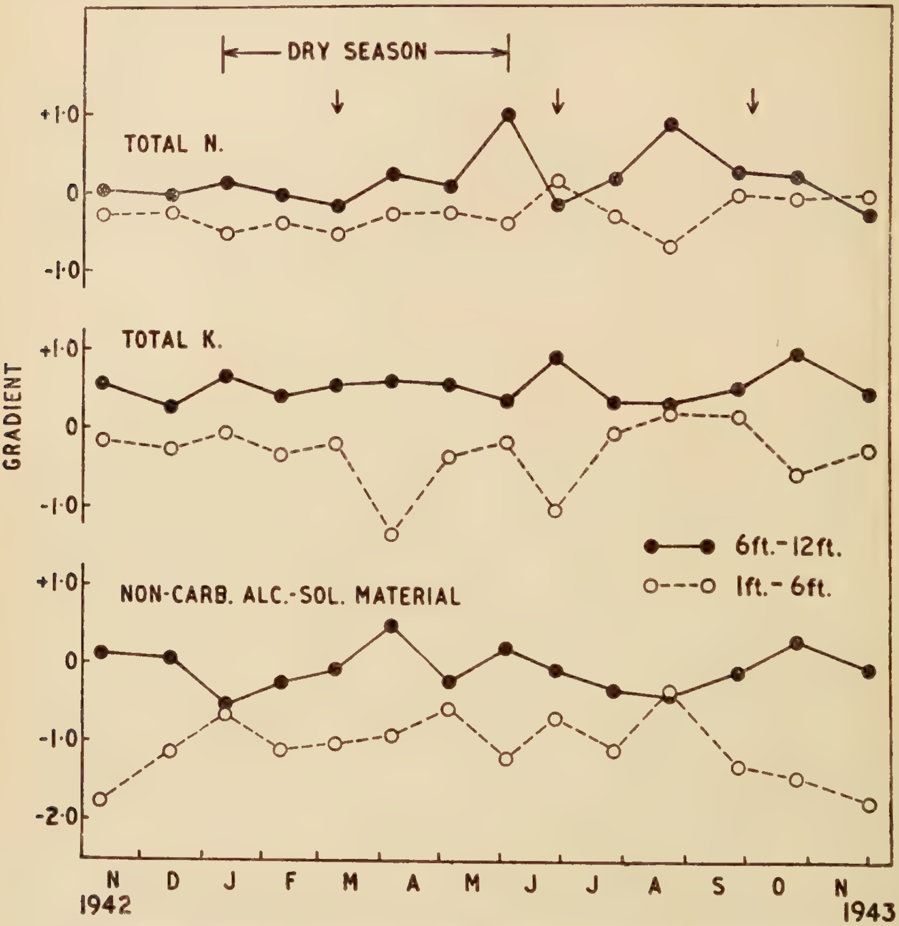


Fig. 2. Seasonal changes in gradient of total nitrogen, potassium, and non-carbohydrate alcohol-soluble material in the 1-ft. to 6-ft. region and the 6-ft. to 12-ft. region in the bark. The downward-pointing arrows indicate the times of occurrence of vegetative flushes.

6 ft. and 12 ft. than the others. In Table II are presented the gradients in this region for the two sets of trees. It will be noted that in the B series the gradient is greater than in the A series nine times out of the fourteen sampling occasions. The gradient in the 1-ft. to 6-ft. region (considered to be mainly due to storage potassium), on the other hand, shows the opposite effect. Here the gradient is greater in the A series eleven times out of the fourteen occasions. This is interpreted as indicating that the storage potassium in the deficient trees falls off more rapidly with increasing height than in the other trees—a conclusion which is in accord with expectation.

TABLE II

Gradients of Total Potassium (g. per 100 g. Water) in Two Regions of the Bark in the Two Sets of Experimental Trees

Date of sampling.	1 ft.–6 ft.		6 ft.–12 ft.	
	A	B	A	B
Nov. 10	−0·42	+0·08	+0·27	+0·83
Dec. 16	−0·28	−0·27	+1·00	−0·50
Jan. 12	+0·73	−0·88	+0·66	+0·61
Feb. 9	−0·21	−0·55	+0·61	+0·15
Mar. 9	−0·03	−0·41	+0·90	+0·11
Apr. 6	−2·84	+0·11	+0·50	+0·64
May 5	+0·05	−0·80	+0·30	+0·78
June 3	+0·27	−0·66	−0·32	+0·97
June 29	−0·69	−1·42	+0·53	+1·23
July 27	+0·44	−0·60	−0·03	+0·65
Aug. 24	+0·72	−0·38	+0·41	+0·16
Sept. 28	+0·59	−0·34	+0·04	+0·93
Oct. 27	−0·43	−0·76	+0·84	+0·99
Dec. 1	−0·70	+0·13	+0·04	+0·77

It would be anticipated that at time of greatest stress or of greatest demand for an element there would be a tendency for positive (dynamic) gradients to become evident or for existing positive gradients to increase. From the gradients of total potassium between the 6-ft. and 12-ft. levels plotted in Fig. 2 it will be noticed that there are five occasions when the positive gradients reach high values: in November, at the beginning of the experimental period when there is a fairly large crop on the trees; in January; in April, during the dry season following a new flush of leaves; in July, at the time of occurrence of a flush; and in October, when there is a large crop on the trees and immediately after a flush has occurred.

Nitrogen

Only analyses of total nitrogen are available and these do not furnish much information about the true dynamic and storage gradients at the different seasons, but as the changes in total nitrogen give indications of a definite trend they will be briefly discussed. The summary in Table I indicates that there is a significant seasonal change in total nitrogen of the bark and that the difference between levels is also significant. The average content of total nitrogen in both samples is lowest at the 6-ft. level (see Appendix, Table III), and there is therefore a change from a negative gradient between 1 ft. and 6 ft. to a positive gradient between 6 ft. and 12 ft. as in the case of potassium. This again appears to be a case of the storage and structural gradient predominating in the lower region and a dynamic gradient predominating in the upper region. No evidence was obtained from the experiment that nitrogen was limiting crop production in any of the experimental trees, but nevertheless it might be expected that a change of gradient would occur in response to increased demand for nitrogen by the crop or foliage or when net uptake from the soil is restricted. It would then be expected that a negative gradient would become positive or that an existing positive gradient would be increased, as demonstrated in the case of potassium. The changes in gradient of total nitrogen with time in the two regions are shown in Fig. 2. In the 1-ft. to 6-ft. region the gradients are all negative with one exception at the end of June when a slight positive gradient exists. The gradients in the 6-ft. to 12-ft. region, on the other hand, are predominantly positive. The gradient in the 6-ft. to 12-ft. region is only slight in the first 5 months of the experiment but increases at the end of the dry season to a maximum (June). Another maximum positive gradient occurs in August.

It is of interest in connexion with changes in nitrogenous reserves to consider further the non-carbohydrate alcohol-soluble material of the bark reported in the preceding paper of this series. This fraction consists in part of soluble nitrogenous substances some of which will be concerned in movement in the bark. Hence changes in this fraction will, to a certain extent, reflect changes both of the storage and dynamic nitrogen. The seasonal drift of this fraction in the two regions is shown in Fig. 2. Between 1 ft. and 6 ft. the gradients are consistently negative as in the case of total nitrogen. At the beginning of the experimental period (when there is a fairly large crop on the tree) the gradient has a large negative value which gradually decreases. The gradient becomes increasingly negative again at the end of the experimental period when a developing crop and a recent flush make demands on the reserves. The gradients in the upper region are both positive and negative. The largest positive gradients occur at the beginning of the experimental period, in April and June (dry-season months) and again in November. All these times correspond with periods of increased utilization of reserves. The first and last are periods of crop demand, while April and June are dry-season months when uptake from the soil is reduced to a minimum and when renewed vegetative growth is occurring.

Phosphorus

The content of total phosphorus in the bark is about half the concentration in the wood (Appendix, Table IV). There is a significant seasonal change but no difference between levels. There does not appear therefore to be any gradient of total phosphorus, and although doubtless some movement of phosphorus takes place in the bark it is masked by the remaining structural phosphorus. The phosphorus of the bark is not correlated with the phosphorus of the wood at any level.

Calcium

The calcium content of the bark is about four times that of the wood (Appendix, Table II) and the gradient is consistently negative throughout the length of trunk examined. The seasons correlation between the calcium of the wood and that of the bark at 1 ft. is high ($r = +0{\cdot}707$, $P = 0{\cdot}01$), which probably indicates considerable cross-leakage of calcium from the vessels of the wood into the bark. No significant correlations were found at other levels.

The Influence of Crop on the Carbohydrate and Mineral Reserves and the Relationships between Potassium, Phosphorus, and Carbohydrates

In the previous paper of this series, using total dry weight as an estimate of crop size, it was possible by means of an analysis of covariance to assess the effect of crop demand on the carbohydrate reserves of the bark and wood. The data permitted an estimate of effects due to seasonal changes (13 degrees of freedom) and residual effect (14 degrees of freedom) at any of the three levels investigated (Humphries, 1947, p. 220). In the case of sucrose, for example, there was a high negative seasonal correlation with crop, pointing to the depletion of sucrose reserves as crop increased. In the case of the residual component also the correlation was negative and significant, indicating that a higher sucrose content was associated with a smaller crop, and it was concluded that carbohydrate (sucrose) was not limiting crop size but that some other factor was responsible.

A similar set of analyses have been carried out in the case of the mineral components, and the correlations between crop and potassium are presented in Table III. The correlations between crop and nitrogen and crop and phosphorus prove to be non-significant. In the case of potassium, in the bark, the seasons correlation has a fairly high negative value at all levels and just fails to reach significance at 6 ft. This indicates a drain on the potassium reserves of the tree with increasing crop. The residual correlations, however, are all *positive* and reach significance at 1 ft. and 6 ft. and also when the total of the three levels are considered (i.e. equivalent to a composite sample of the

Table III

Correlation Coefficient between Crop and Potassium Content

	Bark.		Wood.	
	Seasons.	Residual.	Seasons.	Residual.
1 ft. . .	−0·431	*+0·516*	−0·508	−0·176
6 ft. . .	−0·508	*+0·604*	*−0·625*	+0·107
12 ft. . .	−0·375	+0·373	*−0·652*	+0·093
Sum 3 levels .	−0·465	*+0·643*	*−0·681*	+0·043

Correlations with a significance greater than $P = 0{\cdot}05$ are in italics.

bark up to a height of 12 ft.). This positive correlation indicates that a smaller crop is associated with a smaller content of potassium in the bark, and contrasts with the relationship found between sucrose and crop, suggesting that *potassium is the factor most likely limiting crop size.* In the wood the seasons correlation between crop and potassium is negative at all levels and significant at 6 ft. and 12 ft. and sum of levels, indicating that the potassium reserves of the wood as well as those of the bark are drawn upon by the developing fruits. The residual correlations of wood potassium and crop are low and not significant.

In connexion with the potassium reserves of the cacao tree it may be recalled that, during an investigation into the mineral content of leaves on trees close to the present experimental trees (Humphries, 1940), the leaves of a new flush drew more potassium (44 per cent. of the amount originally present) from the leaves of the next older flush than any other element, an observation which supports the contention that potassium tends to be limiting on the site of these experiments.

In order to see if the picture is substantially altered when the mutual effects of several of the variables are eliminated, the fourth-order correlation coefficients have been calculated. The six variables concerned are sucrose, starch, reducing sugars, potassium, phosphorus, and crop. The first five are expressed as a concentration (g. per 100 g. water) and the latter as g. dry weight. In extracting the partial correlations between sucrose and crop and potassium and crop, interesting relationships between certain other components were revealed and these will be briefly discussed.

The fourth-order seasons correlations are shown in Table IV. The correlation between potassium of the bark and crop is negative at all levels but not significant at any level, just approaching significance at 6 ft. There is a significant negative correlation between potassium of the wood (sum of levels) and crop. The seasons correlation between sucrose and crop is also negative

TABLE IV

Fourth-order Seasons Correlations between Sucrose, Starch, Total Reducing Sugars, Potassium, Phosphorus, and Crop in the Bark and Wood at Three Levels

	Bark.				Wood.			
	1 ft.	6 ft.	12 ft.	Sum.	1 ft.	6 ft.	12 ft.	Sum.
Sucrose × Starch	*−0·720*	*+0·671*	—	*+0·705*	—	—	—	—
Sucrose × K	—	—	—	*−0·618*	—	—	—	—
Sucrose × P	*+0·609*	—	—	—	+0·563	—	—	—
Starch × T.R.S.	*+0·621*	—	*−0·765*	—	—	—	—	—
Starch × K	—	*+0·674*	*+0·733*	*+0·726*	+0·576	—	—	*+0·607*
Starch × P	*+0·809*	—	*+0·738*	*+0·671*	—	—	—	—
T.R.S. × K	—	—	—	—	—	—	—	—
T.R.S. × P	*−0·724*	—	*+0·769*	—	—	—	—	−0·586
Sucrose × Crop	*−0·694*	*−0·632*	—	−0·549	—	—	—	—
K × Crop	—	−0·596	—	−0·538	−0·566	−0·581	—	*−0·700*
Starch × Crop	−0·571	—	—	—	—	—	—	—

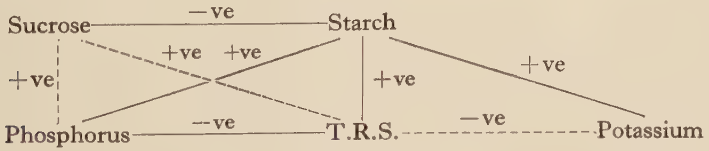
Only correlations with significance greater than $P = 0·1$ are shown. Correlations with significance greater than $P = 0·05$ are in italics.

and significant at the 1-ft. and the 6-ft. levels of the bark. The correlation between starch and crop is not significant at any level. This may be due to the fact that starch in some regions of the bark is not mobilized so that the 'static' starch has a large masking effect. The residual correlation coefficients (Table V) between potassium of the bark and crop are positive at all levels and are significant for the 1-ft. level and for the three levels together. In the wood, however, the correlations are not significant. The residual correlations between sucrose and crop, on the other hand, are negative both in bark and wood and significant at 1 ft., 12 ft., and sum of levels in the bark and at 1 ft. in the wood. Thus the conclusion that potassium limits crop production, while carbohydrate does not, is substantiated.

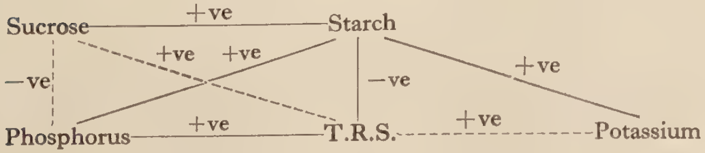
Certain of the other fourth-order seasons correlations shown in Table IV are of interest. Sucrose and starch of the bark show a significant negative correlation at the 1-ft. level but a significant positive correlation at 6 ft. The correlation in the case of the composite sample (sum of levels) is positive and significant. This diametrically opposite relationship between these carbohydrates at the two levels is matched by the case of starch and total reducing sugars. At the 1-ft. level of the bark the correlation is positive and significant while at 12 ft. it is negative and highly significant. The correlation between total reducing sugars and total phosphorus of the bark is negative and significant at 1 ft. and positive and significant at 12 ft. The relationship between starch and phosphorus, on the other hand, is consistently positive and significant at both 1 ft. and 12 ft. and at sum of levels. This is also the case between starch and potassium (significant at 6 ft., 12 ft., and sum of levels in the bark and also at sum of levels in the wood). There are very few significant correlations between components in the wood, but in general they show the same trend as in the bark, although there is no clear evidence of a change from positive to negative values or vice versa at different levels.

The seasons correlations between components of the bark reserves are summarized in the following diagram, where significant correlations between two components are indicated by continuous lines and non-significant correlations by broken lines:

1-ft. level



Higher levels



Although not significant, the change of sign of the correlations is also evident in the cases of Sucrose × Phosphorus and Total Reducing Sugars × Potassium.

The reason for the difference in behaviour at the two levels is not altogether evident and a full explanation is probably not possible with the present data. It may be suggested, however, that the 1-ft. level behaves differently from regions higher in the tree because it is contiguous to the root and possibly, like the root, is predominantly a storage region and hence essentially a region of synthesis.

The positive correlation between starch and potassium is evident both in the bark and the wood. A relationship between starch and potassium has been found in numerous other investigations, but so far no adequate theory of the mechanism of their association is forthcoming. No doubt the positive correlation between starch and phosphorus is connected with the phosphorylation reactions.

Considering now the remainder of the fourth-order residual correlations (Table V), sucrose and starch are negatively correlated in the bark at the 1-ft. level, a general inverse correlation between these two carbohydrates which is to be expected. Sucrose and potassium are positively correlated at the 1-ft. level in the bark, i.e. trees with a high potassium content would tend to have a high sucrose content. On the other hand, trees with a high phosphorus content would tend to have a low sucrose content at the 1-ft. level in the bark. Potassium and phosphorus are positively correlated at the 1-ft. level of the bark.

TABLE V

Fourth-order Residual Correlations between Sucrose, Starch, Total Reducing Sugars, Potassium, Phosphorus, and Crop in the Bark and Wood at Three Levels

	Bark.				Wood.			
	1 ft.	6 ft.	12 ft.	Sum.	1 ft.	6 ft.	12 ft.	Sum.
Sucrose × Starch	*−0·579*	—	—	—	—	—	—	—
Sucrose × K	*+0·648*	—	—	—	—	—	—	*−0·661*
Sucrose × P	*−0·674*	—	—	—	—	—	—	—
Starch × K	—	—	—	—	*−0·577*	—	—	—
Starch × P	—	—	+0·529	—	—	—	—	—
T.R.S. × P	—	*+0·644*	—	*+0·745*	—	—	—	—
K × P	*+0·606*	—	+0·563	+0·525	—	—	—	—
Sucrose × Crop	*−0·879*	—	*−0·630*	*−0·674*	*−0·575*	—	—	−0·545
K × Crop	*+0·761*	+0·502	—	*+0·647*	—	—	—	—
Starch × Crop	−0·553	—	—	—	—	—	—	—
P × Crop	*−0·660*	—	—	—	—	—	—	—

Only correlations with significance greater than $P = 0{\cdot}1$ are shown. Correlations with significance greater than $P = 0{\cdot}05$ are in italics.

It is convenient at this point to sum up the main conclusions from this series of papers concerned with the physiological wilt of cacao fruits. It has been shown that wilt is due to competition between fruits for mineral nutrients. No evidence was found that carbohydrate shortage was a cause of wilt.

Competition was illustrated by the fact that fruits formed early in the season had the greatest chance of survival and of reaching maturity. Fruits formed successively as the season advanced wilted at an increasingly younger age. Fruits formed on thinner and more distant branches had less chance of survival than those formed on the thicker and older parts, i.e. those parts with the greatest reserves of nutrients. It was also shown that competition for nutrients by the foliage may be an important factor in determining survival in young trees. It is concluded that potassium (the element present in greatest amount in the cacao fruit) is chiefly, if not entirely, responsible for diminished crop in the trees investigated.

SUMMARY

The seasonal changes in potassium, phosphorus, nitrogen, and calcium in the bark and wood of the cacao tree at levels of 1 ft., 6 ft., and 12 ft. from the base of the trunk have been followed over a period of 14 months.

There was a significant seasonal variation in potassium and phosphorus of the bark and wood. The seasonal variation in total nitrogen and calcium was significant in the bark but not in the wood.

A negative gradient of potassium was present in the wood and the seasonal changes were correlated with changes in the bark.

There was a negative gradient of calcium in the wood which decreased to a marked extent during the latter part of the dry season. This is held to be evidence that uptake of soil solutes practically ceased at the height of the dry season.

The concentration of potassium in the bark was highest at the 12-ft. level and lowest at the 6-ft. level, so that there was a negative gradient of potassium between 1 ft. and 6 ft. and a positive gradient between 6 ft. and 12 ft. It is assumed that the former is predominantly a structural and storage gradient while the latter is a dynamic transport gradient. The dynamic gradient increased and the storage gradient decreased at times of greatest demands on the potassium reserves. A negative gradient of total nitrogen between 1 ft. and 6 ft. and a positive gradient between 6 ft. and 12 ft. was also found. A similar state of affairs existed in the case of the non-carbohydrate alcohol-soluble material. This fraction, consisting in part of soluble nitrogenous material, will to a certain extent be concerned with movement in the bark. As in the case of potassium, the largest positive gradients occur at periods of active utilization of reserves.

An analysis of covariance between mineral constituents of the bark or wood and crop (expressed as g. dry weight) showed that potassium was depleted by seasonal increase in crop whereas the correlation between potassium and crop at an instant was positive—evidence that potassium limited crop size. Fourth-order correlation coefficients between the variables showed substantially the same picture and also indicated that certain pairs of components were correlated in an opposite manner at different levels.

I am indebted to Dr. D. J. Watson for critically reading the manuscript.

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APPENDIX, TABLE I

Seasonal Trend in Potassium Content (g. per 100 g. Water) of Bark and Wood at Three Levels

	Bark.						Wood.					
	1 ft.		6 ft.		12 ft.		1 ft.		6 ft.		12 ft.	
Sampling date.	A	B	A	B	A	B	A	B	A	B	A	B
Nov. 10	0·29	0·31	0·25	0·32	0·28	0·41	0·48	0·48	0·28	0·42	0·23	0·40
Dec. 16	0·42	0·47	0·39	0·44	0·49	0·39	0·42	0·53	0·30	0·39	0·35	0·35
Jan. 12	0·36	0·55	0·44	0·47	0·50	0·53	0·59	0·50	0·46	0·45	0·41	0·37
Feb. 9	0·48	0·53	0·46	0·48	0·52	0·49	0·53	0·57	0·44	0·57	0·46	0·49
Mar. 9	0·43	0·57	0·43	0·53	0·52	0·54	0·54	0·57	0·47	0·48	0·36	0·41
Apr. 6	0·69	0·46	0·41	0·48	0·46	0·54	0·61	0·54	0·38	0·52	0·41	0·42
May 5	0·53	0·43	0·53	0·35	0·56	0·42	0·50	0·46	0·47	0·64	0·54	0·35
June 3	0·44	0·41	0·46	0·35	0·43	0·44	0·52	0·41	0·48	0·65	0·40	0·54
June 29	0·44	0·50	0·37	0·36	0·42	0·48	0·46	0·58	0·45	0·42	0·41	0·42
July 27	0·45	0·40	0·49	0·34	0·49	0·41	0·57	0·61	0·53	0·47	0·42	0·40
Aug. 24	0·42	0·50	0·49	0·46	0·53	0·48	0·57	0·51	0·62	0·49	0·50	0·39
Sept. 28	0·44	0·39	0·50	0·36	0·50	0·45	0·52	0·58	0·47	0·43	0·43	0·40
Oct. 26	0·44	0·36	0·40	0·28	0·48	0·38	0·50	0·44	0·35	0·36	0·30	0·33
Dec. 1	0·45	0·27	0·38	0·27	0·38	0·35	0·43	0·41	0·37	0·25	0·33	0·24

APPENDIX, TABLE II

Seasonal Trend in Calcium Content (g. per 100 g. Water) of Bark and Wood at Three Levels

	Bark.						Wood.					
	1 ft.		6 ft.		12 ft.		1 ft.		6 ft.		12 ft.	
Sampling date.	A	B	A	B	A	B	A	B	A	B	A	B
Nov. 10	4·60	3·98	3·93	3·92	3·41	3·08	1·11	0·90	1·01	0·80	0·89	1·24
Dec. 16	3·92	3·43	2·95	3·34	1·96	3·51	0·84	0·90	1·01	0·70	0·92	0·78
Jan. 12	4·47	3·15	4·05	3·08	3·15	2·82	0·97	0·87	1·10	0·71	0·81	0·67
Feb. 9	4·21	3·97	3·85	3·39	2·88	3·26	1·29	0·84	0·94	0·82	0·75	0·73
Mar. 9	4·39	4·79	4·03	3·89	3·51	3·38	1·16	1·10	1·38	1·10	1·06	1·03
Apr. 6	3·91	3·96	3·48	3·98	2·91	2·59	0·88	1·09	0·73	0·84	0·82	0·95
May 5	3·65	4·25	3·56	4·07	2·78	3·57	0·83	0·78	1·07	1·10	0·81	0·81
June 3	3·58	4·34	3·31	4·19	3·07	3·30	0·90	0·85	0·77	1·37	0·70	1·19
June 29	3·88	4·54	4·04	4·28	3·52	3·17	0·59	1·19	0·71	1·14	0·56	0·71
July 27	4·31	4·51	3·66	4·33	3·91	3·34	1·01	1·10	1·09	0·89	0·71	0·78
Aug. 24	4·05	3·94	3·25	3·27	3·24	3·47	0·66	1·12	0·90	0·98	0·56	0·83
Sept. 28	4·29	4·28	4·19	3·71	2·82	3·41	1·11	1·03	1·08	0·80	0·79	0·65
Oct. 26	4·21	4·28	3·80	4·19	3·46	3·38	1·06	0·87	0·84	0·85	0·77	0·58
Dec. 1	3·74	4·52	3·83	4·12	2·44	3·61	0·92	0·83	0·61	0·69	0·68	0·60

APPENDIX, TABLE III

Seasonal Trend in Total Nitrogen Content (g. per 100 g. Water) of Bark and Wood at Three Levels

	Bark.						Wood.					
	1 ft.		6 ft.		12 ft.		1 ft.		6 ft.		12 ft.	
Sampling date.	A	B	A	B	A	B	A	B	A	B	A	B
Nov. 10	0·46	0·45	0·43	0·42	0·45	0·41	0·40	0·31	0·37	0·35	0·42	0·43
Dec. 16	0·35	0·40	0·35	0·34	0·32	0·37	0·31	0·40	0·30	0·33	0·30	0·31
Jan. 12	0·44	0·38	0·37	0·34	0·38	0·35	0·36	0·31	0·28	0·31	0·32	0·34
Feb. 9	0·41	0·40	0·37	0·36	0·34	0·37	0·44	0·39	0·47	0·31	0·55	0·47
Mar. 9	0·43	0·44	0·40	0·36	0·37	0·35	0·35	0·37	0·43	0·38	0·41	0·45
Apr. 6	0·43	0·40	0·41	0·36	0·43	0·38	0·47	0·35	0·52	0·35	0·52	0·34
May 5	0·39	0·37	0·32	0·38	0·40	0·31	0·28	0·36	0·29	0·57	0·32	0·44
June 3	0·36	0·41	0·33	0·36	0·37	0·51	0·35	0·35	0·29	0·58	0·34	0·34
June 29	0·37	0·41	0·38	0·43	0·39	0·38	0·34	0·39	0·29	0·42	0·33	0·46
July 27	0·39	0·39	0·34	0·37	0·39	0·36	0·37	0·47	0·35	0·35	0·29	0·42
Aug. 24	0·35	0·43	0·27	0·37	0·40	0·41	0·25	0·36	0·29	0·35	0·30	0·41
Sept. 28	0·38	0·40	0·39	0·38	0·40	0·41	0·37	0·36	0·38	0·35	0·37	0·51
Oct. 26	0·37	0·36	0·33	0·37	0·36	0·37	0·25	0·35	0·35	0·33	0·34	0·35
Dec. 1	0·37	0·41	0·40	0·37	0·34	0·39	0·39	0·35	0·42	0·38	0·36	0·37

APPENDIX, TABLE IV

Seasonal Trend in Phosphorus Content (g. per 100 g. Water) of Bark and Wood at Three Levels

	Bark.						Wood.					
	1 ft.		6 ft.		12 ft.		1 ft.		6 ft.		12 ft.	
Sampling date.	A	B	A	B	A	B	A	B	A	B	A	B
Nov. 10	0·028	0·028	0·025	0·036	0·029	0·035	0·058	0·048	0·044	0·050	0·043	0·051
Dec. 16	0·030	0·026	0·025	0·037	0·027	0·028	0·044	0·053	0·037	0·038	0·041	0·040
Jan. 12	0·029	0·027	0·029	0·029	0·028	0·035	0·059	0·045	0·028	0·034	0·027	0·038
Feb. 9	0·038	0·024	0·040	0·032	0·040	0·037	0·054	0·050	0·047	0·042	0·049	0·048
Mar. 9	0·029	0·032	0·032	0·032	0·031	0·032	0·044	0·046	0·035	0·044	0·036	0·040
Apr. 6	0·029	0·027	0·032	0·026	0·026	0·026	0·049	0·058	0·041	0·043	0·030	0·033
May 5	0·033	0·032	0·034	0·031	0·032	0·029	0·043	0·059	0·048	0·054	0·043	0·043
June 3	0·028	0·033	0·029	0·034	0·030	0·034	0·052	0·071	0·041	0·054	0·045	0·055
June 29	0·029	0·032	0·030	0·034	0·034	0·033	0·057	0·067	0·036	0·067	0·035	0·057
July 27	0·032	0·031	0·033	0·036	0·033	0·029	0·062	0·059	0·054	0·054	0·051	0·080
Aug. 24	0·027	0·031	0·023	0·029	0·025	0·028	0·043	0·055	0·037	0·049	0·034	0·055
Sept. 28	0·031	0·031	0·030	0·030	0·031	0·034	0·065	0·060	0·050	0·055	0·045	0·053
Oct. 26	0·031	0·031	0·030	0·028	0·030	0·031	0·058	0·047	0·044	0·039	0·035	0·035
Dec. 1	0·029	0·027	0·033	0·028	0·030	0·031	0·055	0·048	0·042	0·038	0·044	0·037

Occurrence of Hermaphrodite Flowers and the Development of Female Gametophyte in *Ephedra intermedia* Shrenk et Mey.

BY

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With Plates V and VI and seventeen Figures in the Text

INTRODUCTION

EPHEDRA INTERMEDIA occurs in a state of nature in the north-west Himalayas, Kashmir, and western Tibet at an altitude of 8,000–14,000 ft. above sea-level. Stapf (1889) describes its distribution also from north-east Baluchistan, Afghanistan, and Pamir. It grows in exposed and arid conditions in calcareous soil and among stone crevices in rather inaccessible places. The plant attains a height of about 2 ft. The branches are erect or ascending and the ridges and grooves on them are feebly developed. In Lahul the flowering period was observed by the writer to begin in early June. Pollination occurs in the first week of July and the seeds ripen by the end of September.

The material for the present study was almost exclusively obtained from plants raised from seed in the Government College Botanic Garden at Lahore, Panjab. The seeds were obtained from Chini Range in Bashahr Division through the courtesy of Mr. I. P. Mohan of the Forest Department, to whom the writer wishes to express his thanks. The species has shown a remarkably good growth and has attained a height of more than 3 ft. The plants began to bear flowers after 3–4 years. There has been an alteration in growth and flowering period from that in its natural home in adaptation to the climatic conditions of the Panjab. Here the species begins to sprout out fresh shoots about the last week of February with the arrival of spring. The flowers appear in the middle of March, pollen is shed about mid-April, while the seeds ripen in the last week of June. The other exotic species of the genus that have been cultivated at Lahore, *E. altissima* Desf., *E. saxatilis* Royle, *E. sinica* Stapf, *E. likiagenis* Florin, likewise show a similar growth and flowering period, although the exact time of flowering, ripening of pollen, or maturity of seeds varies somewhat in the different species. This flowering and growth period coincides with that observed for the native species of the genus *E. foliata* Boiss.

This species is of some economic importance. It ranks next to *E. sinica*, *E. gerardiana*, *E. nebrodensis*, and *E. equisetina* as a source of the valuable drug ephedrine. Read and Feng (Chopra, 1933) have estimated the ephedrine content of the species to be nearly 0·4 per cent., while Chopra finds it to be

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in the neighbourhood of 0·2 per cent. The proportion of pseudo-ephedrine, however, is greater than that of ephedrine.

The fixation was mostly done in formaline-acetic-alcohol. This fixative yielded better results than either Bouin's or Nawaschin's as it caused little plasmolysis at the free nucleate embryo-sac stage. Sections were cut from 10 to 20μ thick, depending on different stages. The staining was done with Safranin 'o' in combination with either Fast Green or Gentian Violet.

CONE STRUCTURE

The plants that have developed from seeds are polymorphic. Some bear only male cones, others bear only female cones, while still others are monoecious and bear male, female, and bisexual cones.

Male cone

As in *E. saxatilis*, 2, 3, or 4 cones may be borne at a node. Each cone possesses a stalk up to an inch long. In *E. intermedia* most of the vegetative shoots possess 2 leaves at each node, but there are others more strongly developed which possess 3 leaves at each node. The cones borne on the former type of branch invariably possess a pair of bracts at each node, or in other words they are 'bimerous'. There are 4–5 such pairs decussately arranged, of which the lowest one or two are sterile while the rest are fertile. On the other hand, cones borne on the stouter branches always have bracts borne in 3's at each node, i.e. they are 'trimerous'. There are 4 or 5 such tiers of bracts, of which the lowest one or two are sterile. The bracts of successive tiers alternate.

As usual in the genus, each male flower possesses two perianth leaves arranged anterio-posteriorly enclosing within a central column-like structure or the sporangiophore. The latter bears at the apex 6–8 biolocular sporangia, each of which is shortly stalked. In many cases the sporangiophore itself is bifurcate near the apex and in that case each shank bears 3–4 stalked bilocular sporangia.

Female cone

The female cones are terminal on freshly formed young branches. These branches arise in a whorl of 3–4 at a node in the axils of leaves of previous year's shoots. Like the male cones the female cones may be 'bimerous' or 'trimerous' according as they arise on shoots with 2 leaves at a node or 3 leaves at a node. Each cone bears 3–4 whorls of bracts. Usually, all excepting the uppermost pair of bracts are sterile. The 'dimerous' cone has two ovules, one in the axil of each fertile bract. Often one of these two is abortive. In a 'trimerous' cone there are commonly three ovules, one in the axil of each bract. Similar 'bimerous', 'trimerous', and occasionally 'tetramerous' cones have been observed in *E. foliata* and *E. altissima* growing in the Botanic Garden at Lahore borne on shoots with 2, 3, or 4 leaves at each node respectively.

The two ovules borne on 'bimerous' cone are as a rule free from one another. Not uncommonly the outer integument of the two ovules may be partially or completely fused on the flat side facing each other. If the fusion is complete it gives the appearance of a single large ovule, but the inner integuments of the two ovules are actually free and their respective micropylar tubes are distinct. It appears that this fusion is the result of a common basal growth at the two closely situated growing regions of the outer integument in the ovule primordia. Similar fusion of the outer integuments has also been observed in 'trimerous' cones involving all the three ovules or only two of them.

Such partial or complete union of the outer integuments is not limited only to the present species. It has been observed in *E. saxatilis* and *E. foliata*. It reminds one of a similar condition occurring in the ovules of *Ginkgo biloba* resulting in the formation of 'double ovules' (Mehra, 1938).

While as a rule only the uppermost whorl of bracts is fertile, in rare cases ovules may be borne in the axils of the bracts of the next lower node (Text-fig. 1). Similar features have also been observed in *E. saxatilis* and *E. sinica*. In the latter species both the bracts of the next lower node may bear an ovule each.

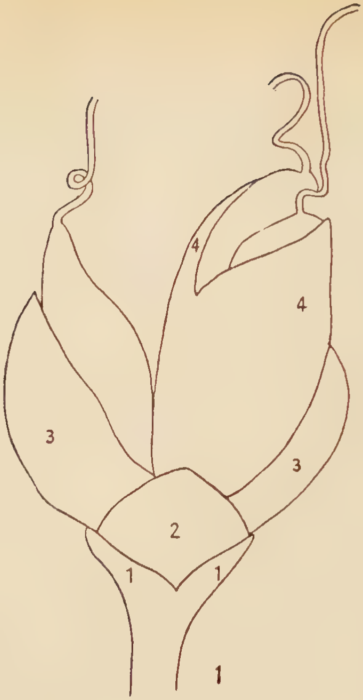
Bisexual cones

Monoecious plants bear male, female, and bisexual cones. There is no regularity in their distribution on the branches. Often two or all the three different types of cones may be borne in a group at the apex of the same branch. In such cases the terminal cone is usually female and those below possess different sexuality. A terminal female and a lateral male is illustrated in Pl. V, Fig. 1 *b*, while a terminal female and a lateral bisexual are shown at *c*. A terminal female, one lateral male, and another lateral bisexual are shown in Pl. V, Fig. 1 *d*. In other cases there may be a terminal female with both laterals of male sex.

In bisexual cones the ovules are borne in the axil of the uppermost pair of bracts; the next pair is usually sterile followed by a pair of bracts bearing male flowers and ending in a pair of sterile bracts (Text-fig. 2). The same is observed in Pl. V, Fig. 1 *a*, but in this bisexual cone only one of the second pair of bracts bears a male flower while the corresponding other is sterile. A 'trimerous' bisexual cone is shown in Text-fig. 3 with five verticils of bracts. The two lowest are sterile followed by a whorl bearing male flowers. This is separated from the uppermost ovule-bearing whorl by a whorl of sterile bracts.

This is not the first record of the occurrence of bisexual cones in the species. A passing reference to this effect is made by Pearson (1929). Bisexual cones are also known in three other species of the genus, namely *E. campylopoda*, *E. americana* var. *Humboldtii*, and *E. trifurca*.

Recently bisexual cones have been observed by the writer in *E. sinica* from plants raised from seeds, although this feature has not so far been recorded.



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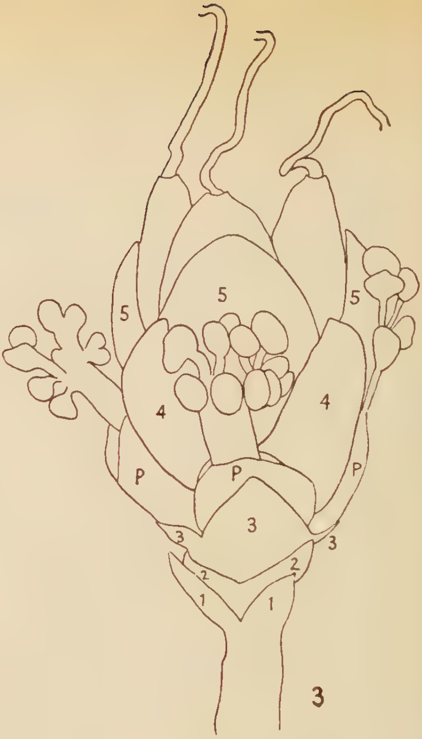

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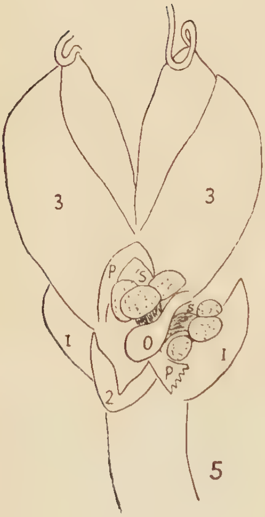

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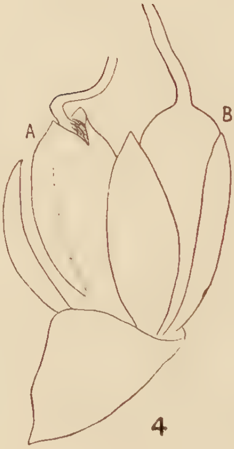

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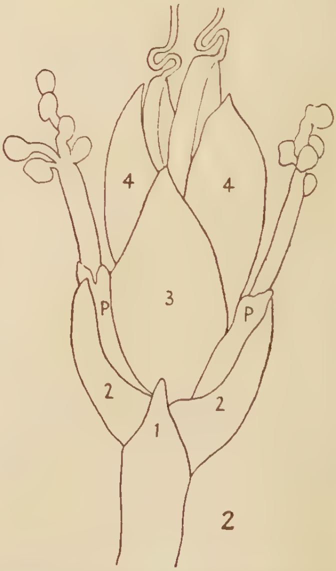

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Fig. 1. A female cone with the uppermost pair of bracts marked ‘4’ fertile, each bearing an ovule in its axil. One bract of the next lower pair is also fertile.

Fig. 2. A ‘bimerous’ bisexual cone, with shortly stalked sporangia on the male flowers.

Fig. 3. A ‘trimerous’ bisexual cone.

Fig. 4. Two ovules that were borne at the apex of a bisexual cone, the one on the left marked ‘A’ with distinctly two lipped outer integument. The one on the right marked ‘B’ had the outer integument cleft to the base on the side facing the reader, on the other side it was bifid.

Fig. 5. A bisexual cone bearing a *hermaphrodite flower*. Of the three decussately arranged pairs of bracts, the lowest marked ‘1’ sterile. Uppermost pair marked ‘3’ each bearing an ovule. Of the middle pair, the one facing the reader marked ‘2’ fertile, while the corresponding on the other side is sterile. In the axil of the former a *hermaphrodite flower* showing two median perianth leaves P, two stamens S, ′S enclosing in the centre an ovule O.

Certain interesting ovules

These deserve special mention. They are somewhat weakly developed and show a striking external resemblance to young male flowers. They are borne either in the axils of uppermost bracts of some bisexual cones or in the axils of the bracts next to the uppermost in some female cones.

It is a well-known fact that a normal ovule in *Ephedra* possesses two integuments, an outer thick and fleshy one completely surrounding the ovule like a collar, and an inner thin and papery one, produced above into a long micropylar tube which is tortuous in *E. intermedia*. Both the integuments are free from one another. The morphology of the outer integument has been in dispute.

The ovules in question show certain features that throw some light on this problem. Two of these borne at the apex of a bisexual cone are drawn on a magnified scale in Text-fig. 4. The outer integument of the ovule on the left marked 'A' is whitish-hyaline, more or less membranous, and distinctly two-lipped at the apex. It appears as if it is formed of two membranous leaf-like structures which are united all along the margins excepting near the apex. Each of these leaf-like structures bears a feebly developed mid-rib. These 'leaves' arise laterally. In the other ovule on the right, marked 'B', the outer integument is completely cleft as far as the base of the ovule on one side (the front side in the diagram), while on the other side it is bifid. The micropylar tube in these ovules is ill developed and is straight or weakly tortuous but the ovule is normal inside.

These ovules irresistibly suggest a homology between the outer integument of the ovule and the perianth leaves of the male flowers. They strongly suggest that the outer integument is in reality of the nature of two fused perianth leaves which have formed a collar-like covering around the ovule proper. These 'perianth leaves', however, arise laterally in contrast to the anterio-posterior arrangement of the perianth in the male flower.

That the outer integument is not really a part of the ovule proper is further shown by the fact that in the abnormal hermaphrodite flowers recorded below for the species, the ovule present in the centre possesses only a single integument—the so-called inner integument.

HERMAPHRODITE FLOWERS

A striking feature in the species is the occasional occurrence of hermaphrodite flowers. Hermaphrodite flowers have not so far been reported in any species of the genus. The rule is the formation of strictly male or strictly female flowers. The hermaphrodite flowers in this species are borne on bisexual cones. Two of these cases may be illustrated.

In Text-fig. 5 is drawn a cone with three pairs of bracts. The lowermost pair marked '1' is sterile, while the uppermost marked '3' bears two ovules, one in the axil of each bract. Of the middle pair, one of the bracts not seen in the figure (being on the opposite side) is sterile while the other bears a *hermaphrodite flower.* The flower was not fully mature and externally appeared like a young unopened male flower covered over by two anterio-posteriorly arranged perianth leaves marked ‘P’. On dissection it showed two stalks, one marked ‘s’ bearing three bilocular sporangia (all of which are not shown) and the other ‘′s’ with two sporangia, one of which is bilocular and the other trilocular. In the centre of the flower is a rudimentary ovule with its only integument produced above into a moderately long micropylar tube.

Another case is illustrated in Pl. V, Fig. 2, from a sectioned cone. In the centre of the flower is observed a poorly developed ovule with its integument produced into a long micropylar tube. On the outside are two stamen stalks bearing in all six bilocular sporangia. One of these stalks is clear in the photograph while the other is in the adjacent section. The whole thing is enveloped on the outside by two perianth leaves. The nucellus of the ovule is yet undifferentiated, while the sporangia are well developed and in these the microspore mother cells are about to be formed.

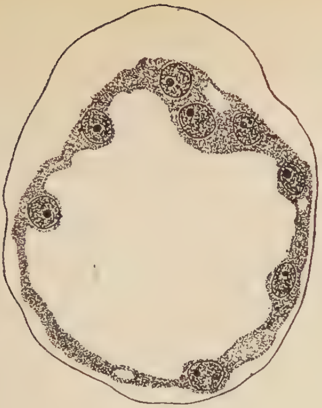
The theoretical significance ascribed to the occurrence of these highly interesting hermaphrodite flowers is dealt with later in discussion.

Development of the Female Gametophyte

The archesporial cell is definitely hypodermal in origin. In *E. saxatilis* the appearance of two hypodermal archesporial cells is not infrequent (Mehra, unpublished). The overlying epidermal cell divides at first by a periclinal wall (Text-fig. 6). This is followed by more periclinal and anticlinal wall forming a multicellular tissue that submerges the archesporial cell deep inside the nucellus. The archesporial cell never cuts off a parietal cell, as has been reported by Maheshwari (1935) in *E. foliata.* It becomes directly transformed into a megaspore mother-cell. The overlying epidermal tissue is thus entirely the product of division of the epidermal cell and not the joint product of division of the epidermal cell and the parietal cell, as maintained by Maheshwari for *E. foliata.*

The megaspore mother-cell undergoes the first reduction division in the usual manner. The leptotene stage has threads chromomeric in nature and the diplotene stage is drawn in Text-fig. 7. The cytoplasm around the enlarged nucleus in the latter stage is fibrillar in appearance. Both the diads undergo a simultaneous division (Text-fig. 8) forming a linear tetrad of four megaspores. Not infrequently the upper diad lags behind and may even degenerate without dividing, while the lower diad invariably forms a row of two megaspores (Text-fig. 9).

The lowest of these megaspores is functional. The earlier stages in the development of the female gametophyte are those characteristic of gymnosperms in general excepting *Welwitschia.* The megaspore enlarges, its nucleus divides into two. A vacuole soon appears in the centre pushing the two nuclei towards the two poles. As the embryo-sac enlarges further, the peripheral



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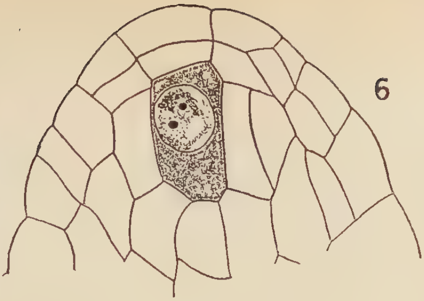

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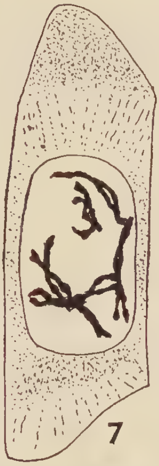

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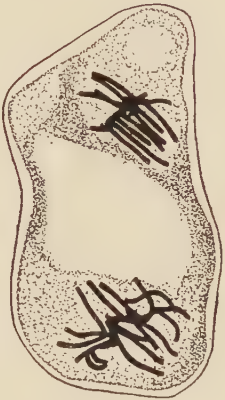

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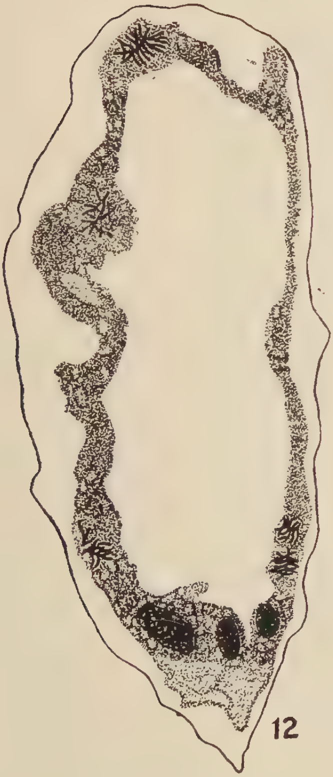

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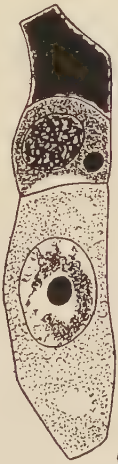

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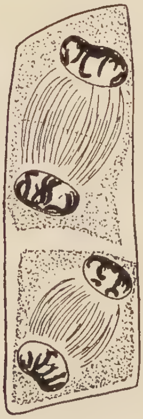

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Fig. 6. Hypodermal archesporial cell. The overlying epidermal cell has divided by a periclinal wall. (×619.)

Fig. 7. Mother cell at diplotene. (×1,310.)

Fig. 8. Both diads dividing simultaneously. (×1,310.)

Fig. 9. Upper diad degenerated. Of the two spores of the lower diad, upper one in the process of degeneration while lower functional. (×1,310.)

Fig. 10. Simultaneous division in the 2-nucleate embryo-sac. (×1,310.)

Fig. 11. A section through a 32-nucleate embryo-sac. (×350.)

Fig. 12. Polarity in the division of the nuclei from 64 to 128-nucleate embryo-sac. Those at the chalazal end still at prophase while the micropylar ones at metaphase. (×220.)

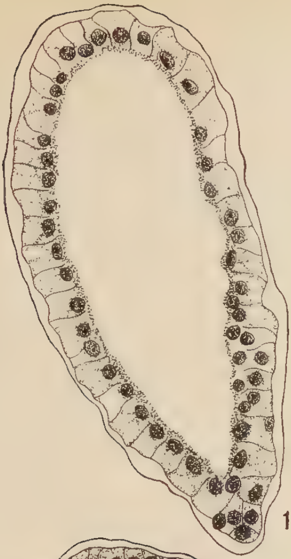
nucellar cells are crushed and partly digested, appearing in sections as darkly stained degenerate masses. The passage from 2- to 4-nucleate stage is seen in Text-fig. 10, both the nuclei being at the metaphase stage. Text-fig. 11 depicts the 32-nucleate stage. The embryo-sac now undergoes a considerable distension in size and then its nuclei divide simultaneously to enter into a 64-nucleate stage. Up to this stage the divisions of the nuclei in the embryo-sac are simultaneous. From now onwards strict regularity is not maintained. In many cases a distinct polarity is visible, the nuclei towards the micropylar end being at a more advanced stage of division than those towards the chalazal end. In an embryo-sac entering into a 128-nucleate stage from 64-nucleate stage, the nuclei towards the micropylar end have entered the metaphase, while the chalazal ones are yet in early prophase (Text-fig. 12).

In the later free-nucleate stages one or more nuclei may become inert and no longer participate in further divisions. These degenerate with obvious repercussions on the total number of nuclei in the succeeding stages, which no longer remain strict theoretical multiples of two.

Wall formation is initiated, as a rule, at 'the 256-nucleate' stage, i.e. after 8 generations of nuclear divisions from the megaspore, when from 210 to 240 nuclei can be counted. But there is considerable variability in this feature. Sometimes walls are observed to be initiated in embryo-sacs in which more than 450 nuclei can be counted, which means after 9 generations of nuclear divisions from the megaspore (Text-fig. 15). This fluctuation in the stage of wall formation in the species is interesting.

A study of literature reveals that the process of wall formation has not been studied in any species of the genus so far. In *Ephedra intermedia* at the close of the free nuclear period a membrane arises on the outer border of the peripheral layer of cytoplasm in which the free nuclei are embedded. This cytoplasm now assumes a fibrillar appearance. Around each nucleus radiating masses of fibres make their appearance as seen from the tangential view. This is followed by the accumulation of cytoplasm in the central region between the adjacent nuclei where the fibrils belonging to the one meet those of the others. In these regions the walls are laid down. Thus polygonal cells are organized as seen in the tangential view. The radial walls of these cells abut directly on the peripheral membrane of the embryo-sac. These cells are at first open towards the interior of the embryo-sac (Text-fig. 15) and at these regions there is dense cytoplasm. When wall formation is complete the embryo-sac is filled by elongated or 'alveolar' cells (Text-fig. 16). Each alveolar developing centripetally cell contains a single nucleus, whose position varies.

When nuclear division has been simultaneous at the last free-nucleate stage, the beginning of wall formation around each nucleus is also simultaneous. In cases where there has been a polarity in division at the last free-nucleate stage, this is continued in the time of wall formation around the nuclei at the two ends of the sac. Thus in Text-fig. 14 the nuclei towards the micropylar end have become enclosed by walls, while no such indication is visible towards the lower end.



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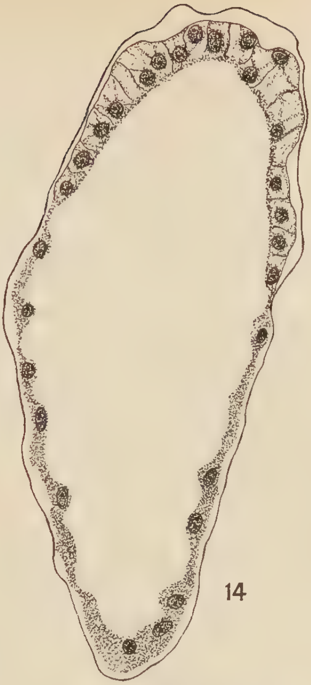

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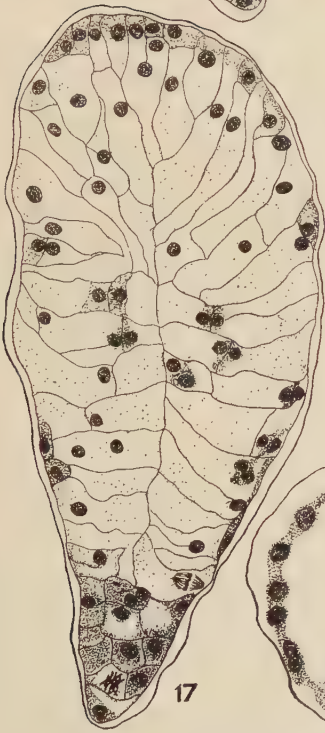

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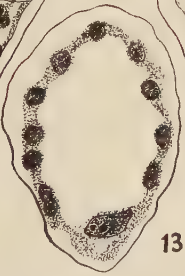

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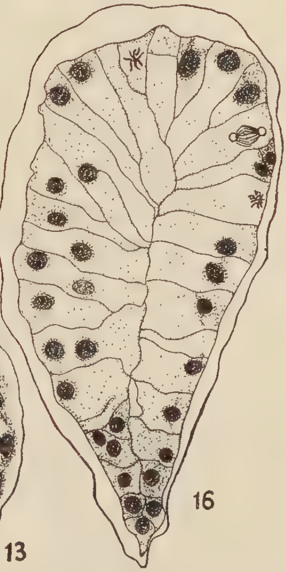

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FIG. 13. A section through a '64-nucleate' embryo-sac (in which 57 nuclei were counted) showing a very large nucleus at the chalazal end probably formed by the fusion of two nuclei. (× 171.)

FIG. 14. Polarity in the laying down of walls in an embryo-sac in which a total of 204 nuclei was counted. (× 171.)

FIG. 15. Wall formation at '512-nucleate' stage (more than 450 nuclei were counted). (× 171.)

FIG. 16. Alveolar method of wall formation. (× 171.)

FIG. 17. Transverse segmentation of alveoli. (× 171.)

After the gametophyte has become completely alveolar the transverse division of the alveoli begins. The place at which the partition is laid down in an alveolus is determined by the position of its nucleus when division occurs. This is variable. In any case the spindle of the mitotic figure is very small and the two daughter nuclei are observed touching the newly formed wall on either side. Further divisions ensue, and every time a wall is laid down so that each cell organized is uninucleate. This further division of the gametophyte is much more active towards the lower end than towards the micropylar end.

The young gametophyte at this stage is conical in shape, broad above and narrow below. It can be differentiated approximately into two halves. The upper half is composed of radially elongated, large, thin-walled hyaline cells with scanty cytoplasm. This gradually merges into the tissue of the lower half of the gametophyte composed of compact, more or less polygonal cells of smaller size with dense contents. The multiplication of cells in both the regions of the gametophyte continues, more so in the lower region than in the upper, with the simultaneous increase in size of the gametophyte as a whole. By the time the archegonia are mature, the lower half of the gametophyte has again undergone a further differentiation into an upper region of polygonal cells undergoing a rather active division, and a basal zone composed of relatively larger cells packed with dense cytoplasm and containing rather large nuclei. The latter cells are apparently haustorial in function, drawing nourishment from nucellar cells below and passing it on to the growing gametophyte. Thus the gametophyte at maturity is differentiated into three zones, an upper fertile zone formed of large hyaline cells, a middle zone of small polygonal cells in an active state of division, and a basal haustorial zone. Viewed under a binocular microscope at this stage, the living teased-out gametophyte shows the upper fertile and translucent region with opaque areas marking the position of archegonia, while the two lower zones are opaque.

Abnormalities

Occasionally, for some unknown reason walls are not laid down in certain areas in some gametophytes at the free nucleate stage. Such areas remain multi-nucleate, or in them at a subsequent period a few walls may be laid down without any regularity. These areas markedly contrast with the rest of the homogeneously developed tissue of the female gametophyte, though they are apparently without significance.

In Pl. VI, Fig. 1, is illustrated a section of a very interesting embryo-sac in the free nucleate stage with nuclei in the process of division. This embryo-sac displayed polarity, the nuclei towards the micropylar end being at telophase while those lower down are at anaphase. Besides the normal nuclei in the process of division one large and highly polyploid nucleus also in a state of division is observed at A. This is lagging behind, the very numerous chromosomes being still at metaphase. Each chromosome is yet double—the daughters having not yet separated. Another section from the same gametophyte showed another nucleus at early telophase which also appeared polyploid. What the fate of such an embryo-sac would be it is difficult to say, but there are indications that it would degenerate.

Diploid Female Gametophytes

While going through the slides it was observed that in certain cases the two ovules belonging to the same cone possessed striking differences in the size of the cells and nuclei of their gametophytes. Not only was there a marked difference in their cell size and nuclear size, but there was also a marked contrast in the size of their respective archegonia. This raised a suspicion that probably the gametophytes with larger cells and nuclei were of polyploid nature. The case was investigated further, and an attempt was made to secure earlier free-nucleate stages of the embryo-sacs with nuclei in the process of division to determine if really polyploid embryo-sacs arise in the species besides the normal ones.

In Pl. VI, Fig. 2, is shown a part of a normal gametophyte at free nucleate stage with nuclei at metaphase. In Pl. VI, Fig. 3, another gametophyte is observed with the nuclei at approximately the same stage of division embedded in the peripheral lining of the cytoplasm. Both of these belong to *Ephedra intermedia*. In none of these can the chromosomes be accurately counted, but the chromosome mass in the latter case appears double that of the former. Obviously the two gametophytes have a different chromosome constitution. The latter appear to be diploid. The formation of such probable diploid female gametophytes is not widespread in the species. They arise only sporadically, while the normal feature is the formation of haploid female gametophytes. Their occurrence, however, is of profound interest in view of the fact that diploid pollen grains to the extent of 2–5 per cent. have previously been recorded by the author in the present species as well as in some other species of the genus, like *E. saxatilis*, *E. altissima*, and *E. foliata* (Mehra, 1946). The method of formation of diploid pollen grains has also been traced in the case of *E. saxatilis* (Mehra, 1947). It has been observed that these diploid pollen grains germinate normally and produce diploid male gametophytes which in turn produce diploid male gametes.

Since the occurrence of diploid pollen grains is fairly widespread in the genus, a similar formation of diploid female gametophytes may be expected to be present in other species of the genus. It is necessary to section a large number of ovules before their presence can be detected.

Discussion

Polymorphism in sex

It is interesting to note that this species exhibits polymorphism in sex. Some plants are either strictly male or strictly female, while others are hermaphrodite bearing male, female, and bisexual cones.

A chromosome study of the species has revealed it to be a tetraploid possessing 28 as the 2*x*-chromosome number. It has been shown to contain 4 basic sets of 7 chromosomes each (Mehra, 1947).

Polyploids which have evolved from simpler unisexual diploids in which sex is determined by sex chromosomes are now known to display polymorphism in sex (similar to that exhibited by the present species) depending upon the ratio of X chromosomes to autosomes. The occurrence of sex chromosomes has not yet been established in any species of the genus *Ephedra* by cytological analysis, but the strict sexuality displayed by the male and female plants and the occurrence of hermaphrodite ones in individuals of the present species suggests that these individuals may be sex-controlled by the ratio of X chromosome to autosomes in their nuclear constitution. The species may have come from *immediate* unisexual diploid ancestors in which sex was governed by sex chromosomes. It seems that the sex chromosomes are not strikingly different morphologically from the autosomes in the genus *Ephedra*.

Hermaphrodite flowers and their significance

The three genera of the Gnetales, *Ephedra*, *Welwitschia*, and *Gnetum*, show characters in common, and yet there are striking differences in a number of morphologic and embryologic characters. Because of these differences they have been placed in three distinct families, namely, *Ephedraceae*, *Welwitschiaceae*, and the *Gnetaceae*.

One of these differences is in the structure of flowers. In *Ephedra* the flowers are typically unisexual. The male flower consists of two perianth leaves arranged anterio-posteriorly. Enclosed within is a central column-like structure, the sporangiophore, bearing at the apex a variable number of bilocular sporangia. The sporangiophore is supplied within by *two* vascular strands. The number of sporangia in many species is 8, although in others the number may be reduced to 3 or even 2. In *E. foliata* the number of sporangia is commonly 3. The sporangia may be sessile or in some species shortly stalked. Not infrequently the sporangiophore is bifurcate at the apex, the bifurcation sometimes extending very deep down. This has been noted by Thoday and Berridge (1912) in *E. distachya* and *E. nebrodensis* and by the writer in the present species. In such cases each shank is supplied with a single vascular strand. The total number of sporangia in these is evenly distributed on the two branches.

The structure of the male flower in *Gnetum* is on the same plan as in *Ephedra*. The perianth forms a cup-like envelope. From the centre of this projects an axial structure bearing two apical sporangia. Like *Ephedra*, this axial sporangiophore is traversed by *two* vascular strands.

In *Welwitschia*, on the other hand, the male flower is built very differently. It is pseudo-hermaphrodite. There are 4 perianth leaves, 2 outer smaller lateral, and 2 inner larger anterio-posterior. There are 6 stamens within, arranged in a ring. Each of these is supplied with a *single* vascular strand. These are united in the lower region to form a tube but are free above, each bearing terminally a trilocular sporangium. In the centre of the tube is a sterile ovule with its only integument produced above into a long micropylar tube characteristic of the Gnetales. In these sterile ovules the apex of the micropylar tube is flaring.

Apparently there seems to be a striking dissimilarity between the male flower of *Ephedra* or *Gnetum* on the one hand and that of *Welwitschia* on the other, and it has frequently been doubted if they are at all related.

The abnormal hermaphrodite flowers observed in *Ephedra intermedia* serve as a link connecting the normal male flower of *Ephedra* with that of *Welwitschia*. These abnormal flowers possess two perianth leaves arranged anterio-posteriorly as in the normal male flower. Enclosed within are two stamens bearing bilocular sporangia at their apices, while the centre is occupied by an ovule possessing a single integument drawn out above into a micropylar tube. Such a flower structure is remarkably similar in plan to that of *Welwitschia* except for certain differences of detail such as the number of perianth leaves and the number of stamens. These observations not only bring the Ephedraceae and the Welwitschiaceae close together but show that the modern Gnetales as a whole may have come from a stock with hermaphrodite flowers. In *Welwitschia*, relics of the ancestral condition are still retained with the pseudo-hermaphroditic condition of the male flower as a normal feature. Such a condition is normally lost in the Ephedraceae and the Gnetaceae, although, as in the present species, it may show itself exceptionally. The derivation of the male flower seen in the present-day forms of the genus *Ephedra* seems to have been from the original hermaphrodite flowers by the loss of the female part within and the fusion of two stamen stalks to form a central solid axial column. Thus the axial column is supplied by two vascular strands, whereas each stamen in *Welwitschia* possesses a single vascular strand. Also often the sporangiophore is more or less deeply cleft into its component parts. Sporangiophores which are bifurcated at the apex obviously represent a primitive condition as suggested by Thoday and Berridge (1912).

Nature of the outer integument of the ovule

The ovule in *Ephedra* possesses two integuments, an outer which is thick and fleshy and an inner thin membranous produced above into a long micropylar tube. The outer integument is free from the inner. In *E. intermedia* it is supplied by two vascular strands which are laterally disposed. The inner integument, on the other hand, is free from vascular supply and is fused with the nucellus excepting towards its micropylar end.

The morphological nature of the outer integument has been a matter of some discussion in the past. Certain abnormal and rather weakly developed ovules described and illustrated in the text suggested to the writer that the morphological nature of the outer integument is that of fused perianth leaves. The so-called outer integument in these ovules was thin and looked very much like the perianth of the male flowers. At its apex there were two distinct lobes which suggested that the outer integument is formed by the fusion of two 'leaf-like' structures. In another case the fusion between the two 'leaves' was completely absent on one side, while on the opposite side bifid nature was evident. That the outer integument is not a part of the ovule proper is further shown by the fact that it is entirely absent in the ovules present in the centre of the hermaphrodite flowers observed in *E. intermedia.*

Flower structure of the probable ancestors of the Gnetales

The flower in the ancestral stock of the Gnetales probably possessed two whorls of perianth leaves with two members in each whorl, a variable number of stamens (whose stalk was supplied with a single vascular strand) arranged in a ring bearing sporangia at the apices, and in the centre an ovule possessing a single integument which was fused with the nucellus in the lower region and was elongated above to form a micropylar tube. From this ancestral hermaphrodite flower by further modifications flower structure in *Welwitschia*, *Ephedra*, and *Gnetum* seems to have been derived along the following lines.

In *Welwitschia* the male flower has retained almost the original condition excepting for the fact that the ovule has become sterile—a trend towards the direction of unisexuality. The female flower is derived by the loss of inner anterio-posterior whorl of perianth leaves and the stamen ring. The two perianth leaves of the outer lateral whorl have united along their margins to form the outer envelop of the female flower.

In *Ephedra* in the derivation of the male flower the outer lateral whorl of perianth leaves has disappeared along with the ovule in the centre. The two anterio-posteriorly arranged members of the inner whorl have remained. At the same time the two stamens have undergone concrescence along the sides, giving rise to an axial column-like structure. This is supported by the fact that the stamen stalk of the present-day flowers is supplied by two vascular strands, while each stamen in *Welwitschia* possesses a single vascular bundle. In the female flower the inner anterio-posterior whorl of perianth leaves together with the two stamens have disappeared. The two lateral perianth leaves have fused along their margins, giving rise to the so-called outer integument as in *Welwitschia.*

In *Gnetum* the derivation of the male flower is on similar lines to that in *Ephedra.* In the female flower, on the other hand, the members of the outer whorl of perianth have coalesced to form the outermost envelope and the members of the inner whorl have united to form the middle envelope around the ovule. The ovule, strictly speaking, possesses only one integument which forms the micropylar tube. This has occurred together with the disappearance of the staminal whorl.

SUMMARY

Sex polymorphism is described in *Ephedra intermedia.* Some plants are strictly male or strictly female, while others are hermaphrodite, bearing male, female, and bisexual cones. Since *E. intermedia* is tetraploid it is suggested that display of sex may be governed in the individuals by the ratio between X chromosome and autosomes as is the case in other polyploids derived from unisexual diploids.

The hermaphrodite flowers are built on the same plan as the pseudo-hermaphrodite male flowers of *Welwitschia*. There are two perianth leaves arranged in a median plane followed inwards by two stamens each bearing three or four bilocular sporangia with an ovule in the centre possessing single integument produced upwards into a micropylar tube. The significance of this discovery is discussed.

The archesporial cell in *E. intermedia* and *E. saxatilis* is hypodermal in origin and becomes directly transformed into a megaspore mother-cell. The usual linear tetrad of megaspores is formed although sometimes the upper diad does not divide. The lowest megaspore is functional.

The development of the embryo-sac follows the general plan in this genus, although some variation is observed in the time of wall formation and in the occurrence of diploid or polyploid nuclei.

Occasionally diploid female gametophytes appear besides the normal haploid ones.

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EXPLANATION OF PLATES

Illustrating P. N. Mehra's article 'Occurrence of Hermaphrodite Flowers and the Development of Female Gametophyte in *Ephedra intermedia* Shrenk et Mey.'

PLATE V

Fig. 1. Cones from a monoecious plant of *Ephedra intermedia*. (× about 2½.) *a*. Bisexual cone. *b*. A cluster of two cones, terminal female and lateral male. *c*. A cluster of two cones, terminal female and lateral bisexual. *d*. A cluster of three cones, terminal female and lateral male, and the other bisexual.

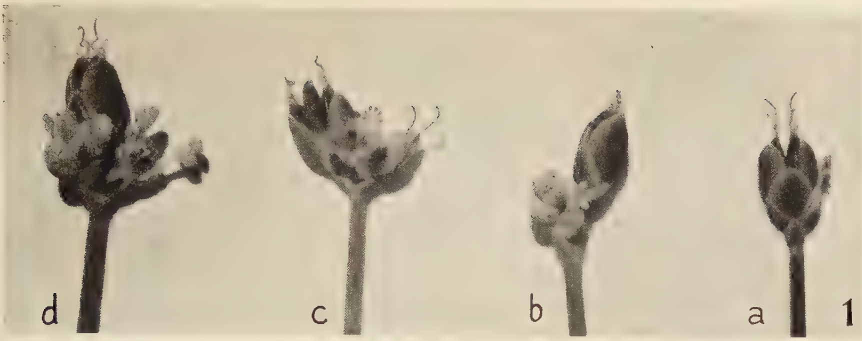
Fig. 2. V.S. through a hermaphrodite flower of *E. intermedia*. Note the ovule in the centre with its only integument elongated above to form a micropylar tube T. Outside it on the right a stamen is visible bearing a bilocular sporangium. The stalk of the second stamen is in the succeeding section, but one of its sporangium is visible. Outside are the two perianth leaves marked PP.

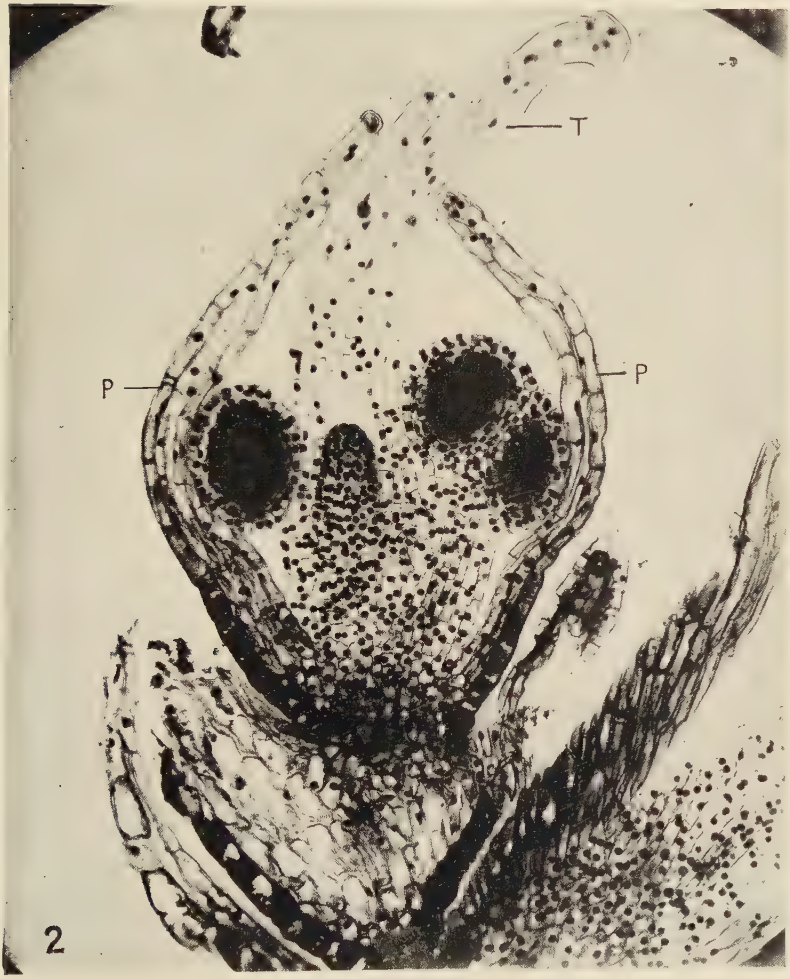
PLATE VI

Fig. 1. Section through free-nucleate embryo-sac with nuclei in division stage. There is polarity; those towards the upper micropylar end are at telophase while chalazal ones are at anaphase. At A is observed an exceptional highly polyploid nucleus at metaphase with a very large number of chromosomes.

Fig. 2. Section through a normal haploid embryo-sac at free-nucleate stage with nuclei at metaphase.

Fig. 3. Section through another embryo-sac at free-nucleate stage with nuclei at the same stage as in the previous figure. The metaphase clump here is double that in the former. This is probably diploid.





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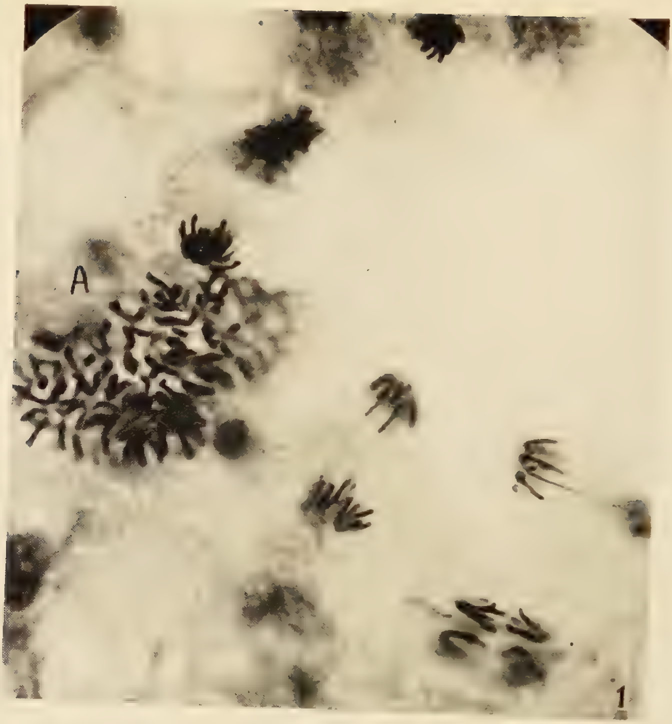

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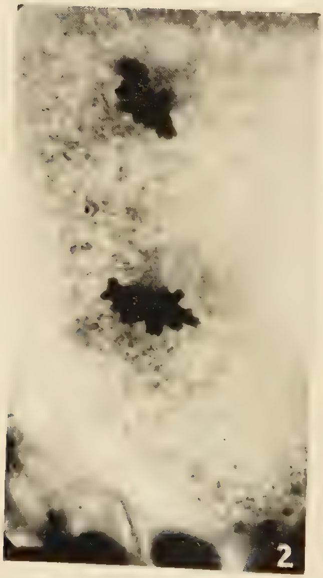
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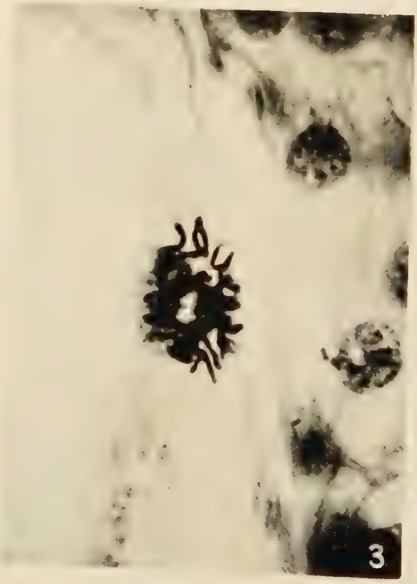
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P. N. MEHRA

The Effect of the Plant Growth Regulator, 4-Chloro 2-Methyl Phenoxyacetic Acid, on the Mineral and Nitrogen Contents of Plants

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INTRODUCTION

TEMPLEMAN and Sexton (1946) have shown that certain concentrations of the aryloxyacetic acids, notably 4-chloro 2-methyl phenoxyacetic acid (M.C.P.A.), produce a differential effect on the growth of different species and that this may be utilized as a means of selective weed control.

Rhodes and Templeman (1947) investigated the nature of this differential response in spring oats (variety 'Victory'), corn chamomile (*Anthemis arvensis*), and rape (*Brassica campestris*), and reported that the sodium salt of 4-chloro 2-methyl phenoxyacetic acid markedly interfered with the potassium metabolism of the most susceptible species, rape, while having little or no effect on that of corn chamomile and oats. In the present paper these preliminary results are presented in greater detail together with a further investigation of the effect of sodium 4-chloro 2-methyl phenoxyacetate on the mineral and nitrogen contents of another susceptible species, tomato (*Solanum lycopersicum*), and the influence of the potassium status of the plant on its response.

PART I

The Effect of Sodium 4-Chloro 2-Methyl Phenoxyacetate on the Mineral Content and Growth of Species having Varying Susceptibilities to Its Action

Experimental details

As a result of field experience, rape and spring oats were selected as examples of susceptible and resistant crops, and corn chamomile for intermediate response.

Seedlings of the three species were grown from seed in small pots containing silver sand. Two hundred pots for each species were sown, the number of seeds per pot varying with the size of seed in the following manner:

Rape (*Brassica campestris*)	16 seeds per pot
Corn chamomile (*Anthemis arvensis*) . . .	approximately 30 seeds per pot
Spring oats (var. 'Victory')	12 seeds per pot

[Annals of Botany, N.S. Vol. XIV, No. 54, April, 1950.]

The pots were lightly watered daily until the seedlings were showing above the sand and thereafter 40 ml. of nutrient solution was applied per pot, once or twice daily according to weather conditions, the nutrient containing 100 p.p.m. N, 50 p.p.m. P_2O_5, 200 p.p.m. K_2O, 40 p.p.m. MgO, together with other essential elements in lower concentrations, and having a pH of 6.

When the seedlings were well established they were thinned to four seedlings per pot and, for each species, 150 of the most uniform pots were selected for experimentation.

In each species the pots were allotted at random into five sets, A, B, C, D, and E, each consisting of 30 pots. The five sets from a given species were placed in five parallel rows on a trolley so that they could be moved as required. Five treatment solutions, the highest of which was estimated to be the maximum concentration which the most susceptible species could survive, were prepared by dilution with the nutrient solution from a stock solution of 0·01 g. per cent. of the sodium salt of M.C.P.A. and allotted to the sets of pots as follows:

A . . .	0 p.p.m.	sodium	4-chloro	2-methyl phenoxyacetate
B . . .	0·01 p.p.m.	,,	,,	,,
C . . .	0·05 p.p.m.	,,	,,	,,
D . . .	0·10 p.p.m.	,,	,,	,,
E . . .	0·50 p.p.m.	,,	,,	,,

From the time when the seedlings were 3 to 4 weeks old the treatment solutions were applied from a graduated separating funnel to the sand surface of the appropriate pots at the rate of 40 ml. per pot either once or twice daily according to weather conditions. Application of the treatments began on the following dates:

Rape	May 9, 1946
Corn chamomile	May 11, 1946
Oats	May 1, 1946

At approximately weekly intervals one sample consisting of five pots was withdrawn at random from each treatment within a given species. The roots were washed free from sand, blotted to remove surface moisture, and separated from the 'tops'. The samples were then killed and dried at approximately 100° C. and the dry weights of the samples from five plants were determined. In some samples (p. 188) a further subdivision of 'tops' was made.

The dried 'tops' from the five plants taken from each species at a given sampling time were then bulked, ground to a powder in a Christie & Norris 5-in. mill, re-dried at 100° C., and taken for determination of percentage compositions in regard to potassium, phosphorus, and magnesium. The estimations of magnesium and potassium were done spectrographically, while the phosphorus was determined chemically. Details of the analytical methods are given in the appendix.

Owing to the amount of work involved it was not possible to examine all samples from each species, but the mineral determinations were made on the more important ones, namely, 0, 0·1, and 0·5 p.p.m. M.C.P.A.

Results

i. *Rape* (*Brassica campestris*). This species was highly susceptible. Chlorosis of the foliage, swelling of the hypocotyl and root-tips, presence of adventitious roots, and a less fibrous appearance of the roots were marked after only 7 days' treatment at 0·5 p.p.m. A similar effect was noticeable on the roots after 35 days with the concentration so low as 0·05 p.p.m. After 5 weeks' treatment at 0·5 p.p.m., the root system consisted almost entirely of bulbous root initials which had arisen from the main root.

ii. *Corn chamomile* (*Anthemis arvensis*). This species was moderately susceptible. Characteristic changes in the morphology of the roots and hypocotyl appeared after 17 days of treatment at 0·5 p.p.m. There was a marked development of adventitious roots just behind the apices of the lateral roots, these adventitious roots, being in one plane, giving a feather-like appearance to the parent root.

iii. *Spring oats* (*variety* '*Victory*'). This species was most resistant. The roots were unaffected until 28 days after beginning the 0·5 p.p.m. treatment, when it was noted that the adventitious roots which had developed near the apices of the main roots were very short and had bulbous swellings at their apices.

The mean dry weight per plant of the roots and 'tops' at each sampling time are tabulated in Table I, while the K_2O, P_2O_5, and MgO contents of the bulked samples of 'tops' expressed as a percentage of the bulked dry weights of the 'tops' are given in Table II.

Discussion

The visual observations show quite clearly the differential response of the three species to sodium 4-chloro 2-methyl phenoxyacetate. For example, after only 7 days of treatment there was an effect on the root system of rape at 0·5 p.p.m., but for corn chamomile this was not observed until 17 days and for oats 28 days. Again, rape was the only species to show thickening of the lateral roots at 0·05 p.p.m.

The dry-weight figures also bring out the differences in species sensitivity. Thus for the 'tops' there is a significant reduction at 0·5 p.p.m. for rape at all sampling times after the first, while there is also a significant reduction at 0·1 p.p.m. at the sixth sampling time. For corn chamomile similar reductions are observed at 0·5 p.p.m., but the differences are much smaller and there is no effect at 0·1 p.p.m. For oats there is no reduction in dry weight at any sampling time, even at the highest concentration of M.C.P.A. (Actually there is a just significant reduction at 0·5 p.p.m. at the third sampling time, but since there is no indication of such reduction at any subsequent sampling time it would appear to be fortuitous.)

For the roots, only rape is affected at 0·5 p.p.m., differences being found at the third and later sampling times. It is particularly interesting to note that the dry weight of the 'tops' are reduced before any reduction in root dry weight is observed, although, as previously described, the morphology of the root has already been modified.

TABLE I

Mean Dry Weight per Plant—'Tops' and Roots

'TOPS'

Sampling time.	Period of treatment (days).	Mean dry weight per plant.					Standard error of single sample.	Coefficients of variation.	Significant difference between means.
		0 p.p.m. M.C.P.A.	0·01 p.p.m. M.C.P.A.	0·05 p.p.m. M.C.P.A.	0·10 p.p.m. M.C.P.A.	0·5 p.p.m. M.C.P.A.			
Rape (seed sown April 15)								%	
May 17	7	0·09	0·09	0·10	0·10	0·12	0·03	29·1	N.S.
,, 24	14	0·28	0·26	0·28	0·27	0·12	0·06	24·6	0·08
,, 31	21	0·57	0·64	0·57	0·57	0·15	0·10	20·1	0·13
June 7	28	0·70	0·71	0·73	0·62	0·18	0·10	17·0	0·13
,, 14	35	1·33	1·02	1·14	1·11	0·21	0·19	19·6	0·25
,, 21	42	1·64	1·30	1·54	1·07	0·32	0·20	17·1	0·26
Corn Chamomile (seed sown April 17)									
May 21	10	0·04	0·04	0·03	0·04	0·04	0·01	35·2	N.S.
,, 28	17	0·08	0·08	0·09	0·07	0·02	0·03	47·3	0·04
June 4	24	0·26	0·33	0·32	0·29	0·15	0·07	27·7	0·10
,, 11	31	0·52	0·50	0·54	0·57	0·32	0·12	23·6	0·15
,, 19	39	0·90	0·68	0·84	0·76	0·57	0·21	27·6	N.S.
,, 25	45	1·12	0·99	1·13	0·93	0·58	0·22	23·2	0·29
Oats (seed sown April 17)									
May 8	7	0·16	0·15	0·11	0·15	0·12	0·04	30·3	N.S.
,, 15	14	0·15	0·15	0·18	0·19	0·23	0·05	28·3	N.S.
,, 21	20	0·37	0·33	0·39	0·34	0·26	0·05	14·4	0·06
,, 29	28	0·70	0·71	0·68	0·69	0·66	0·13	18·4	N.S.
June 5	35	1·16	1·08	1·17	1·08	1·06	0·15	13·2	N.S.
,, 13	43	2·11	1·75	2·01	2·09	1·70	0·25	13·0	N.S.
ROOTS									
Rape (seed sown April 15)									
May 17	7	0·06	0·06	0·04	0·05	0·05	0·02	41·4	N.S.
,, 24	14	0·07	0·05	0·06	0·07	0·06	0·02	26·5	N.S.
,, 31	21	0·11	0·11	0·11	0·15	0·10	0·06	48·6	N.S.
June 7	28	0·19	0·19	0·19	0·14	0·08	0·05	34·6	0·07
,, 14	35	0·39	0·33	0·30	0·38	0·14	0·07	23·3	0·10
,, 21	42	0·45	0·38	0·49	0·39	0·16	0·12	33·8	0·17
Corn Chamomile (seed sown April 17)									
May 21	10	0·02	0·02	0·01	0·02	0·01	0·01	29·5	N.S.
,, 28	17	0·04	0·04	0·05	0·05	0·04	0·02	57·0	N.S.
June 4	24	0·09	0·12	0·13	0·12	0·09	0·03	31·4	N.S.
,, 11	31	0·15	0·14	0·18	0·22	0·11	0·05	33·9	N.S.
,, 19	39	0·25	0·20	0·21	0·22	0·20	0·06	28·2	N.S.
,, 25	45	0·24	0·18	0·26	0·20	0·18	0·02	11·6	N.S.
Oats (seed sown April 17)									
May 8	7	0·16	0·17	0·23	0·10	0·16	0·07	42·4	N.S.
,, 15	14	0·16	0·18	0·18	0·13	0·20	0·05	26·9	N.S.
,, 21	20	0·22	0·20	0·21	0·19	0·30	0·06	25·0	0·08
,, 29	28	0·29	0·33	0·34	0·33	0·45	0·07	22·1	N.S.
June 5	35	0·55	0·50	0·48	0·61	0·80	0·17	28·2	N.S.
,, 13	43	0·71	0·70	0·76	0·74	0·77	0·11	15·0	N.S.

N.S. = Not significant.

TABLE II

K_2O, P_2O_5, and MgO Contents of Bulked Samples of 'Tops' expressed as Percentage of the Dry Weights

Sampling time.	Dry weight of bulked sample (g.).			K_2O content as % of bulked dry weight			P_2O_5 content as % of bulked dry weight			MgO content as % of bulked dry weight		
				A	D	E	A	D	E	A	D	E
	0 p.p.m. M.C.P.A.	0·1 p.p.m. M.C.P.A.	0·5 p.p.m. M.C.P.A.	0 p.p.m. M.C.P.A.	0·1 p.p.m. M.C.P.A.	0·5 p.p.m. M.C.P.A.	0 p.p.m. M.C.P.A.	0·1 p.p.m. M.C.P.A.	0·5 p.p.m. M.C.P.A.	0 p.p.m. M.C.P.A.	0·1 p.p.m. M.C.P.A.	0·5 p.p.m. M.C.P.A.
Rape (seed sown April 15)												
May 17	0·47	0·52	0·61	3·0	2·8	2·5	1·9	—	1·6	0·9	0·7	0·5
,, 24	1·61	1·33	0·60	5·8	5·9	4·5	1·5	1·5	1·4	0·8	0·8	0·8
,, 31	2·87	2·84	0·73	7·2	7·2	2·5	1·0	1·2	1·0	0·8	0·9	1·1
June 7	3·48	3·08	0·92	12·3	10·0	4·8	1·1	1·4	1·3	2·1	2·8	6·3
,, 14	6·65	5·57	1·06	10·0	10·0	3·4	1·3	1·3	1·1	1·3	1·6	2·6
,, 21	8·22	5·36	1·60	9·2	8·5	3·6	1·1	1·1	1·1	1·0	1·7	2·6
Corn chamomile (seed sown April 17)												
May 21	0·22	0·20	0·19	11·8	11·1	12·2	—	—	—	0·9	1·0	1·1
,, 28	0·40	0·36	0·12	12·9	11·6	10·6	1·8	1·7	—	0·8	0·7	1·5
June 4	1·30	1·43	0·74	10·6	11·1	8·4	1·2	1·3	1·4	0·7	0·7	0·9
,, 11	2·60	2·67	1·59	10·8	6·0	6·0	1·5	1·5	1·6	0·9	0·9	1·2
,, 19	4·50	3·80	2·87	8·7	10·2	9·2	1·2	1·2	1·2	0·9	1·1	1·1
,, 25	5·62	4·67	2·91	8·7	9·2	7·6	0·8	1·0	1·1	1·1	1·1	1·0
Oats (seed sown April 17)												
May 8	0·80	0·75	0·58	9·4	7·2	7·1	2·4	2·3	2·3	0·5	0·7	0·6
,, 15	0·77	0·95	1·16	7·1	5·6	4·8	2·4	2·2	2·0	0·6	0·5	0·4
,, 21	1·85	1·69	1·30	7·0	6·0	5·6	1·9	1·9	2·0	0·7	0·6	0·5
,, 29	3·50	3·42	3·28	8·6	8·1	6·0	1·8	1·4	1·4	0·6	0·6	0·6
June 5	5·82	5·42	5·29	6·1	6·9	5·9	1·6	1·4	1·4	0·4	0·5	0·5
,, 13	10·50	10·44	8·45	7·4	8·3	5·0	1·3	1·4	1·4	0·4	0·4	0·5

The fact that the above differential responses were produced by applications of M.C.P.A. to the roots only clearly indicates that they are due to physiological differences between the three species and not to any differences in morphology of the aerial portions.

From the data of Table II it is seen that there are no differences due to M.C.P.A. treatment in P_2O_5 content of the tops of any of the three species. A very different picture is seen for the K_2O contents, where there are marked reductions in K_2O content of the rape 'tops' at 0·5 p.p.m. at all sampling times. No such obvious or consistent differences are observed for the other two species. Since rape also showed the greatest depression in dry weight of the tops, it is clear that the reduction in K_2O content of the rape tops is even greater than the percentage figures of Table II would suggest.

For magnesium content there is obviously no depression corresponding to that found for potash in any species. If anything, there appears to be a greater MgO content of the rape 'tops' at 0·1 p.p.m. and 0·5 p.p.m. M.C.P.A. at the fourth and subsequent sampling times.

Rape, the most susceptible species, thus appears to differ from the other species in two ways:

(i) Examination of the control figures in Table II shows that the K_2O content of the tops of corn chamomile and of oats does not vary much throughout the period of treatment, although in the case of the former the dry weight increased by about 27 times and in the case of the latter by about 10 times. The K_2O content of the untreated rape, on the other hand, rose gradually during the first 28 days with increasing dry weight and then remained roughly the same although the dry weight continued to increase.

(ii) Treatment of rape with 0·5 p.p.m. M.C.P.A., besides giving a considerable reduction of dry-matter production, has also retained the K_2O content at about the low figure of the first sample right throughout the period of treatment.

Thus it is seen that the sodium salt of 4-chloro 2-methyl phenoxyacetic acid markedly interferes with the potassium metabolism of the most susceptible species. The nature of this interference and its possible bearing on the causes of the selective phytocidal action are further investigated in Part 2.

PART 2

The Effect of 4-Chloro 2-Methyl Phenoxyacetic Acid on the Mineral and Nitrogen Contents of a Susceptible Species, Solanum Lycopersicum (Tomato)

The specific depression of the potash content of the tops of rape described in Part 1 was followed by a more extensive examination of the effect of M.C.P.A. on the mineral and nitrogen contents of another susceptible species, *Solanum lycopersicum* (tomato), and the influence of the external potassium supply on the response.

Experimental details

On September 2, 1947, 100 uniform seedling tomatoes (variety 'Stonor's M.P.') were selected from a group of 3-week-old plants which had been grown in sand under normal soil-less culture conditions in an unheated glasshouse. These plants were potted into 48-size pots with silver sand and allotted into five blocks of 20 plants each. Within each block all treatments were distributed to the plants at random. Owing to limitations of space and the necessity for employing non-technical assistance in the daily application of the treatments, no randomization of the position of plants within a block was possible. The results, therefore, have not been submitted to statistical analysis, but they have been interpreted on the basis of large differences shown by the treatment means.

For the following 17 days the plants in each block were given five nutrient solution treatments in which the ratio of K_2O/Na_2O was varied as follows:

K(1)	37 p.p.m. K_2O	606 p.p.m. Na_2O
K(2)	67 ,,	566 ,,
K(3)	117 ,,	496 ,,
K(4)	217 ,,	371 ,,
K(5)	372 ,,	206 ,,

These levels of potassium and sodium were selected so as to maintain, as far as possible, a constant total salt concentration for all nutrient solutions, while leaving the composition unaltered with respect to other ions. Of the amounts of sodium and potassium supplied, 206 p.p.m. Na_2O and 17 p.p.m. K_2O were present in the main water supply.

In preparing the different nutrient solutions, a basal stock solution was made up for subsequent dilution at 1 in 100. This solution contained:

$MgSO_4, 7H_2O$	246 g.
$NH_4H_2PO_4$	81 g.
K_2SO_4	37 g.
Minor elements concentrate (1)[1]	1 litre

Distilled water to 10 litres.

50 ml. of the stock solution were diluted with 4·75 litres of tap-water containing one of the following mixtures of potassium nitrate and sodium nitrate to give the treatment solutions K(1)–K(5) which were adjusted to pH 6 by the addition of sulphuric acid as required:

For K(1)	2·735 g. $NaNO_3$	Nil KNO_3
,, K(2)	2·460 g. ,,	0·320 g. ,,
,, K(3)	2·000 g. ,,	0·855 g. ,,
,, K(4)	1·125 g. ,,	1·920 g. ,,
,, K(5)	Nil ,,	3·600 g. ,,

Two applications, each of 50 ml., were given daily per pot.

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[1] The Minor elements concentrate (1) contained the following salts dissolved in 10 litres of distilled water:

Lithium chloride ($LiCl \cdot 2H_2O$)	0·28 g.	Nickel sulphate ($NiSO_4$ $6H_2O$)	0·56 g.
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0·56 ,,	Cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$)	0·56 ,,
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0·56 ,,	Titanium oxide ($Ti\ O_2$)	0·56 ,,
Boric acid (H_3BO_3)	6·12 ,,	Potassium iodide (KI)	0·28 ,,
Aluminium sulphate ($Al_2(SO_4)_3$)	0·56 ,,	Potassium bromide (KBr)	0·28 ,,
Stannous chloride ($SnCl_2 \cdot 2H_2O$)	0·28 ,,	Sodium silicate (Na_2SiO_3)	4·33 ,,
Manganese sulphate ($MnSO_4 \cdot 4H_2O$)	9·75 ,,	Ferric citrate ($FeC_6H_5O_7$ $3H_2O$)	64·20 ,,

On September 19, after 17 days of the above treatments, there were no visible differences. The sodium 4-chloro 2-methyl phenoxyacetate treatments were then superimposed on the potassium-level treatments to give the following concentrations of M.C.P.A. in the nutrient solutions:

(*a*) 0 p.p.m. sodium 4-chloro 2-methyl phenoxyacetate.
(*b*) 0·1 p.p.m. ,, ,, ,, ,,
(*c*) 0·3 p.p.m. ,, ,, ,, ,,
(*d*) 0·9 p.p.m. ,, ,, ,, ,,

From September 30 until the completion of the experiment, one daily application of 90 ml. of nutrient was applied per pot.

By October 2 the plants had grown considerably and the concentration of M.C.P.A. was therefore increased to the following levels:

(*a*) 0 p.p.m. sodium 4-chloro 2-methyl phenoxyacetate.
(*b*) 0·15 p.p.m. ,, ,, ,, ,,
(*c*) 0·45 p.p.m. ,, ,, ,, ,,
(*d*) 1·35 p.p.m. ,, ,, ,, ,,

The plants were subjected to the M.C.P.A. treatments for a total period of 21 days, during which time visual observations were made. At the end of this period, on October 10, the plants were harvested. Each plant was divided into three portions, 'root', 'lower stem', and 'tops', obtained in the following manner:

'Root': Stem cut at insertion of cotyledons. Roots carefully washed free from sand and blotted dry.

'Lower stem': A second cut was made in the stem at a point 7 in. above the insertion of the cotyledons. Since the lowest leaves on this portion were rather damaged owing to the surface application of nutrient, all the leaves (a maximum of three) were removed from the lower stem and discarded.

'Tops': This portion comprised all the stem and leaves above the 'lower stem' portion.

The tissues were killed in an oven at 95° C. and the dry weights were then determined by drying at 90° C.

The dried samples were ground to a powder in a Christie & Norris 5-in. mill and then placed in 4-oz. screw-cap jars until required for analysis.

Details of the analytical methods are given in the appendix.

Results

(*a*) *Visual observations*

19/9/47.	Commenced to apply M.C.P.A. treatments.
24/9/47.	Lower leaves of all plants receiving the highest level (*d*) of M.C.P.A. showed epinasty in the region of the pulvinus.
1/10/47.	Lower leaves of all plants receiving M.C.P.A. treatment (*c*) showed similar effects to those previously observed for treatment (*d*). The leaves in these two treatments appeared somewhat 'emaciated' and the lower leaves were yellowish.

In treatment (*d*) the plants receiving the three lowest potash levels, K(1), K(2), and K(3), had young leaves which showed a slight but definite chlorosis of the leaf margins.

Adventitious root initials were apparent as bulges on the lower 4 in. of stem of all plants receiving treatment (*d*).

2/10/47. First truss just visible on all plants.

8/10/47. Opening of flowers of first truss delayed in plants receiving highest M.C.P.A. treatment. Thus only 3 out of 20 plants had open flowers in treatment (*d*) as against 17, 16, and 16 for treatments (*a*), (*b*), and (*c*) respectively.

Growth of the axillary buds was progressively inhibited with increasing concentration of M.C.P.A. Thus the shoot in the axil of the leaf immediately below the first truss measured as follows:

M.C.P.A. treatment (*a*) 3–4 in. in length.
,, ,, (*b*) 1–3 in. in length.
,, ,, (*c*) less than 1 in. in length.
,, ,, (*d*) No axillary shoot.

The plants receiving M.C.P.A. treatments (*b*), (*c*), and (*d*) appeared 'spindly', this effect being more marked with increasing concentration of M.C.P.A.

Adventitious root initials were apparent as bulges on the stems of plants receiving M.C.P.A. treatment (*c*), similar to those previously noted for treatment (*d*).

10/10/47. All plants harvested.

(*b*) *Dry weights and analytical data.* For all treatments the mean values of the five replicates with respect to dry weight and total nitrogen, K_2O, P_2O_5, MgO, and CaO contents expressed as percentages of the dry weights are given in Table III. From these data the mean values of the absolute weights of total nitrogen, K_2O, P_2O_5, MgO, and CaO, have been calculated and these are presented in Table IV.

It is clear that, in general, the trends shown by increasing M.C.P.A. over all potash levels are similar, as are those shown by increasing potash over all M.C.P.A. levels. To facilitate interpretation, therefore, the data have been condensed in Table V, where the mean values of dry weight and total nitrogen, K_2O, P_2O_5, MgO, and CaO expressed as percentages of the dry weights, over all M.C.P.A. levels are shown for increasing levels of potash, together with the mean values over all potash levels for increasing M.C.P.A. concentrations.

The data of Table IV for the absolute amounts of total nitrogen, K_2O, P_2O_5, MgO, and CaO have been condensed in a similar manner in Table VI.

Visual examination of the spectrographic plates showed no consistent or obvious differences between treatments in iron, copper, manganese, or sodium in the tops or roots.

Discussion

The general effect of M.C.P.A. treatment on the mineral status of the tomato plant is very similar to that previously noted for another susceptible species, rape. Thus the data of Table III show that M.C.P.A. treatment results in a markedly lower K_2O content of the 'tops', this effect becoming progressively greater with increasing concentration of M.C.P.A. A similar condition is found for the 'lower stems'. In the roots, however, which were

Table III

Mean Values of Dry Weights and Total N, K_2O, P_2O_5, MgO, and CaO Contents expressed as Percentages of the Dry Weights

	K(1)				K(2)				K(3)				K(4)				K(5)			
	(a)	*(b)*	*(c)*	*(d)*	*(a)*	*(b)*	*(c)*	*(d)*	*(a)*	*(b)*	*(c)*	*(d)*	*(a)*	*(b)*	*(c)*	*(d)*	*(a)*	*(b)*	*(c)*	*(d)*
TOPS																				
Dry weight (g.)	1·67	1·74	1·46	0·74	1·80	1·64	1·52	0·93	2·08	1·80	1·53	1·05	2·22	1·95	1·48	1·02	2·47	2·35	1·57	0·97
Total N content (as % of dry wt.)	4·32	3·61	3·72	3·73	3·97	3·83	3·67	3·96	3·87	3·65	3·72	3·66	3·70	3·86	3·71	3·67	3·62	3·57	3·71	3·53
,, K_2O ,, ,, ,,	5·9	5·4	4·5	3·1	6·4	5·8	4·7	3·7	7·4	7·3	5·8	4·6	8·2	7·7	6·8	4·6	8·3	8·0	7·6	6·0
,, P_2O_5 ,, ,, ,,	1·36	1·38	1·35	1·29	1·38	1·38	1·30	1·12	1·48	1·37	1·30	1·06	1·39	1·27	1·15	1·17	1·36	1·22	1·13	0·99
,, MgO ,, ,, ,,	1·7	2·1	2·0	1·9	1·8	1·8	2·0	2·0	1·5	1·6	1·9	1·9	1·6	1·7	1·7	2·0	1·5	1·6	1·6	1·5
,, CaO ,, ,, ,,	1·1	1·3	1·3	1·2	1·4	1·3	1·3	1·5	1·2	1·2	1·2	1·3	1·1	1·1	1·5	1·2	1·1	1·3	1·0	1·0
LOWER STEMS																				
Dry weight (g.)	0·80	0·97	0·88	0·90	0·87	0·87	0·89	1·13	0·93	0·93	0·74	0·95	1·00	0·92	0·92	1·07	1·09	0·98	1·03	1·18
Total N content (as % of dry wt.)	1·97	1·69	1·60	1·58	1·48	1·52	1·67	1·55	1·50	1·69	1·43	1·53	1·62	1·57	1·29	1·53	1·55	1·48	1·43	1·54
,, K_2O ,, ,, ,,	4·5	2·8	2·9	2·4	5·2	4·9	3·3	3·0	6·6	5·5	4·9	3·8	7·3	6·7	5·8	6·0	7·8	7·3	7·7	6·9
,, P_2O_5 ,, ,, ,,	1·00	0·91	0·90	0·97	0·98	0·92	0·90	0·96	0·92	0·97	0·88	0·88	1·08	0·98	0·81	0·91	1·12	0·91	0·81	0·81
,, MgO ,, ,, ,,	1·5	1·7	1·6	1·8	1·4	1·4	1·5	1·5	1·3	1·3	1·5	1·4	1·2	1·2	1·2	1·2	1·0	1·2	1·1	0·9
,, CaO ,, ,, ,,	0·6	0·6	0·7	0·7	0·7	0·5	0·6	0·5	0·5	0·6	0·5	0·6	0·5	0·5	0·5	0·5	0·5	0·5	0·4	0·4
ROOTS																				
Dry weight (g.)	0·72	0·89	0·74	0·72	0·83	0·61	0·86	0·71	0·81	0·72	0·67	0·59	0·94	0·74	0·84	0·70	0·96	0·88	1·01	0·96
Total N content (as % of dry wt.)	2·40	2·18	2·56	2·94	2·92	2·23	2·42	3·04	2·25	2·38	2·76	3·00	2·18	2·09	2·58	3·46	1·99	2·38	2·76	3·56
,, K_2O ,, ,, ,,	2·2	2·2	2·8	4·1	1·9	2·7	3·1	4·4	2·5	3·2	4·2	4·9	3·2	3·8	4·6	5·6	4·3	4·9	5·7	6·4
,, P_2O_5 ,, ,, ,,	0·77	0·76	0·79	1·06	0·79	0·78	0·87	1·11	0·78	0·78	0·92	1·04	0·81	0·81	0·84	1·08	0·77	0·80	0·90	1·16
,, MgO ,, ,, ,,	1·1	1·2	0·9	0·9	1·2	1·0	0·9	0·9	1·2	1·0	0·9	0·8	1·0	0·9	0·9	0·8	1·0	0·8	0·8	0·7
,, CaO ,, ,, ,,	0·5	0·5	0·5	0·5	0·5	0·5	0·4	0·5	0·6	0·5	0·5	0·6	0·5	0·5	0·5	0·5	0·4	0·5	0·4	0·5

TABLE IV

Mean Values of Dry Weights and Absolute Amounts of Total N, K_2O, P_2O_5, MgO, and CaO

	K(1)				K(2)				K(3)				K(4)				K(5)			
	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)
TOPS																				
Dry weight (g.)	1·67	1·74	1·46	0·74	1·80	1·64	1·52	0·93	2·08	1·80	1·53	1·05	2·22	1·95	1·48	1·02	2·47	2·35	1·57	0·97
Absolute amount total N (mg.)	70	62	54	27	71	63	55	37	80	65	57	38	82	74	54	37	89	82	58	34
,, ,, K_2O ,,	94	91	65	22	113	97	73	34	156	130	88	47	183	145	104	45	201	191	120	62
,, ,, P_2O_5 ,,	22	24	20	9	25	23	20	11	31	25	20	11	31	25	17	12	33	29	18	10
,, ,, MgO ,,	28	35	30	14	32	30	30	19	32	29	30	20	36	33	25	20	38	37	25	16
,, ,, CaO ,,	19	22	19	9	26	22	19	14	24	21	19	13	24	21	22	12	28	30	16	10
LOWER STEMS																				
Dry weight (g.)	0·80	0·97	0·88	0·90	0·87	0·87	0·89	1·13	0·93	0·93	0·74	0·95	1·00	0·92	0·92	1·07	1·09	0·98	1·03	1·18
Absolute amount total N (mg.)	15	16	14	14	16	13	13	17	14	16	11	14	16	14	12	17	17	15	15	18
,, ,, K_2O ,,	37	26	25	21	44	44	29	33	62	52	35	35	74	61	55	64	84	71	78	79
,, ,, P_2O_5 ,,	8	9	8	9	9	8	8	11	9	9	7	8	11	9	8	10	12	9	8	9
,, ,, MgO ,,	12	17	14	17	12	12	13	16	12	13	12	13	12	10	11	13	11	12	11	10
,, ,, CaO ,,	5	6	6	6	6	4	5	6	5	6	4	6	5	5	5	5	5	5	4	5
ROOTS																				
Dry weight (g.)	0·72	0·89	0·74	0·72	0·83	0·61	0·86	0·71	0·81	0·72	0·67	0·59	0·94	0·74	0·84	0·70	0·96	0·88	1·01	0·96
Absolute amount total N (mg.)	16	19	19	21	19	14	21	21	18	17	18	18	20	15	22	25	19	21	27	34
,, ,, K_2O ,,	16	18	21	30	16	16	27	31	21	24	28	29	30	26	38	39	40	44	57	59
,, ,, P_2O_5 ,,	5	7	6	8	7	5	8	8	6	6	6	6	7	6	7	8	7	7	9	11
,, ,, MgO ,,	8	11	7	7	10	6	8	6	9	7	6	5	9	6	7	6	9	8	8	7
,, ,, CaO ,,	3	4	3	4	4	3	3	4	5	4	3	3	4	4	4	4	4	4	4	5

not previously examined, the reverse is true, the K_2O content increasing rapidly with rising M.C.P.A. concentration.

The visual observations and mean plant dry weights indicate that the effect of M.C.P.A. on the tomato plant is not alleviated by an increase in the supply of potash to the roots, even though this additional potassium leads to a higher over-all potassium status within the tissues. The M.C.P.A. symptoms are clearly not due to a simple deficiency of potassium in the 'tops', since it may be seen from Table III that the K_2O content of the 'tops' of the plants receiving the highest M.C.P.A. treatment in conjunction with the highest potash supply is about equal to that of the controls of the lowest potash group.

From the data of Table V it is seen that the very marked effect of M.C.P.A. on the K_2O content of the 'tops' and roots is unique. Similar but very much less obvious trends, however, are shown by the P_2O_5 and total-N contents.

It is of interest to note that M.C.P.A. has no influence over the CaO content of any tissue and little if any effect on the MgO content. Similarly, visual assessment of the spectrographic plates could not detect any consistent differences in the Na, Mn, Ca, or Fe content of the tissues due to M.C.P.A. treatment. By contrast, the K_2O differences were readily apparent.

The changes in K_2O content of the tissues cannot be accounted for by the different dry weights between treatments, since there is little effect of M.C.P.A. during the period of the experiment on the dry weight of the roots, while the dry weight of the tops is markedly depressed. The following possibilities might account for the observed potash changes:

(*a*) An interference with the transport of potassium to the 'tops'.
(*b*) A redistribution of potassium from the 'tops' to the roots.
(*c*) A change in the rate of potassium uptake by the roots, superimposed on (*a*) or (*b*).

In the absence of data for the K_2O status of the plants at the beginning of M.C.P.A. treatment, it is not possible to decide between the first two possibilities.

Some information on the effect of M.C.P.A. on potassium uptake can, however, be obtained by a consideration of Table VII where mean values of the total N, K_2O, P_2O_5, MgO, and CaO contents over all potash levels, for the whole plants, have been calculated from the data of Table VI. It is thus seen that the K_2O content of the whole plant is markedly and progressively reduced with increasing M.C.P.A. concentration. This large depression is peculiar to potassium, no effect being seen for CaO and MgO, and only very slight depressions for P_2O_5 and total N. It seems likely, therefore, that M.C.P.A. brings about a specific inhibition of uptake of potassium. The reduction in potassium uptake by the roots may be due to the following:

(*a*) A real reduction of potassium uptake without any reduction in the absorbing surface of the roots.
(*b*) A reduction of active absorbing surface owing to morphological changes in the root system.

TABLE V

(a) Mean Values of Dry Weights and Total N, K_2O, P_2O_5, MgO, and CaO Contents for increasing Potash Level over All M.C.P.A. Concentrations

	Dry weight (g.)			Total N content (as % of dry wt.)			K_2O content (as % of dry wt.)			P_2O_5 content (as % of dry wt.)			MgO content (as % of dry wt.)			CaO content (as % of dry wt.)		
	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.
Increasing potash ↓ K(1)	1·38	0·89	0·77	3·85	1·71	2·54	4·7	3·2	2·8	1·34	0·94	0·84	1·9	1·7	1·0	1·2	0·6	0·5
K(2)	1·47	0·94	0·75	3·86	1·59	2·49	5·2	4·1	3·0	1·29	0·94	0·89	1·9	1·4	1·0	1·4	0·6	0·5
K(3)	1·62	0·90	0·70	3·73	1·54	2·60	6·3	5·2	3·7	1·30	0·91	0·88	1·7	1·4	1·0	1·2	0·6	0·5
K(4)	1·67	0·98	0·80	3·73	1·50	2·58	6·8	6·5	4·3	1·25	0·95	0·88	1·8	1·2	0·9	1·2	0·5	0·5
K(5)	1·84	1·07	0·95	3·61	1·50	2·67	7·5	7·4	5·3	1·17	0·91	0·91	1·6	1·0	0·8	1·1	0·5	0·5

(b) Mean Values of Dry Weights and Total N, K_2O, P_2O_5, MgO, and CaO Contents for increasing M.C.P.A. Concentrations over All Potash Levels

	Dry weight (g.)			Total N content (as % of dry wt.)			K_2O content (as % of dry wt.)			P_2O_5 content (as % of dry wt.)			MgO content (as % of dry wt.)			CaO content (as % of dry wt.)		
	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.
Increasing M.C.P.A. ↓ (*a*)	2·06	0·94	0·86	3·90	1·69	2·22	7·2	6·3	2·8	1·39	1·02	0·78	1·6	1·3	1·1	1·2	0·6	0·5
(*b*)	1·89	0·93	0·77	3·70	1·59	2·25	6·8	5·5	3·4	1·32	0·94	0·79	1·8	1·4	1·0	1·2	0·6	0·5
(*c*)	1·51	0·90	0·82	3·71	1·44	2·61	5·9	4·9	4·1	1·24	0·86	0·86	1·8	1·4	0·9	1·3	0·6	0·5
(*d*)	0·94	1·04	0·74	3·71	1·54	3·22	4·4	4·4	5·1	1·17	0·91	1·09	1·9	1·4	0·8	1·2	0·6	0·5

TABLE VI

(*a*) *Mean Values of Dry Weights and Absolute Amounts of Total N, K_2O, P_2O_5, MgO and CaO for increasing Potash Level over all M.C.P.A. Concentrations*

	Dry weight (g.)			Absolute amount of N (mg.)			Absolute amount of K_2O (mg.)			Absolute amount of P_2O_5 (mg.)			Absolute amount of MgO (mg.)			Absolute amount of CaO (mg.)		
Increasing potash ↓	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.
K(1)	1·38	0·89	0·77	52	15	19	66	27	21	19	8	6	27	15	8	17	6	4
K(2)	1·47	0·94	0·75	56	15	19	79	38	23	19	9	7	28	13	7	20	6	4
K(3)	1·62	0·90	0·70	60	14	18	105	46	25	21	8	6	28	12	7	19	5	4
K(4)	1·67	0·98	0·80	62	15	21	119	63	33	21	9	7	28	12	7	20	5	4
K(5)	1·84	1·07	0·95	66	16	25	143	78	50	22	10	9	29	11	8	21	5	4

(*b*) *Mean Values of Dry Weights and Absolute Amounts of Total N, K_2O, P_2O_5, MgO and CaO for increasing M.C.P.A. Concentrations over All Potash Levels*

	Dry weight (g.)			Absolute amount of N (mg.)			Absolute amount of K_2O (mg.)			Absolute amount of P_2O_5 (mg.)			Absolute amount of MgO (mg.)			Absolute amount of CaO (mg.)		
Increasing M.C.P.A. ↓	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.	Tops.	Lower stems.	Roots.
(*a*)	2·06	0·94	0·86	79	16	18	152	61	25	29	10	7	33	12	9	24	5	4
(*b*)	1·89	0·93	0·77	69	15	17	131	51	26	25	9	6	33	13	7	23	5	4
(*c*)	1·51	0·90	0·82	56	13	21	90	44	34	19	8	7	28	12	7	19	5	4
(*d*)	0·94	1·04	0·74	35	16	24	42	46	37	11	9	8	18	14	6	11	6	4

The former appears more likely since the effect seems to be confined to the potassium ion.

It is most improbable that the altered K_2O content of the tissues can be the result of changes in their histology alone, since from Table V (*b*) it may be seen that in the lower stem, where adventitious root initials are present, the K_2O content of the tissue is still greatly diminished as compared with the controls. However, the much smaller observed changes in total N and P_2O_5 contents of the various portions may conceivably be due to this cause.

TABLE VII

Mean Values of Dry Weights and Total N, K_2O, P_2O_5, MgO, and CaO Contents of Whole Plants expressed as Percentages of the Dry Weights for increasing M.C.P.A. Concentration over all Potash Levels

	M.C.P.A. concentration.	Dry weights of whole plants (g.).	Total K_2O content (as % of dry wt.).	Total P_2O_5 content (as % of dry wt.).	Total MgO content (as % of dry wt.).	Total CaO content (as % of dry wt.).	Total N content (as % of dry wt.).
Increasing M.C.P.A. ↓	(*a*)	3·86	6·2	1·19	1·4	0·9	2·93
	(*b*)	3·59	5·8	1·11	1·5	0·9	2·81
	(*c*)	3·23	5·2	1·05	1·5	0·9	2·79
	(*d*)	2·72	4·6	1·03	1·4	0·8	2·76

It may be concluded, therefore, that M.C.P.A. applied to the roots of a susceptible species, tomato (variety Stonor's M.P.) in sub-lethal amounts, has a specific effect on the potassium metabolism of the plant, causing a marked diminution of the K_2O content of the 'tops' and lower stems and a marked accumulation of potassium in the roots, the K_2O content of the plant as a whole being greatly lowered. These effects appear to be due to a specific inhibition of uptake of potassium by the roots combined with either an interference with the transport of potassium from the roots to the tops or with a redistribution of potassium from the tops to the roots. Further investigation of these effects may help in determining the mode of action of M.C.P.A. and may also lead to a better understanding of the role of potassium in plant nutrition.

It is possible, therefore, that the effects of the sub-lethal concentrations of M.C.P.A. on the mineral nutrition of a susceptible species, described in this paper, may be observed in a more severe form in susceptible species sprayed under field conditions with the usual phytocidal amounts of M.C.P.A. and that they may contribute to the death of the plant. The rapidity with which these changes in K_2O content of the tissues under the influence of M.C.P.A. can occur and the manner in which they are brought about is now being investigated, together with their relationship to the changes in carbohydrate metabolism reported by Mitchell (1946) as being induced by a closely related compound, 2-4-dichlorophenoxyacetic acid.

APPENDIX

Methods of analysis. Owing to the intention to examine the tissues with regard to their content of a fairly wide range of elements and because of the small amounts of material available, it was decided that only total nitrogen and phosphate could be satisfactorily determined by chemical methods. Total nitrogen was determined by the usual semi-micro Kjeldahl technique using sodium sulphate and selenium during digestion and boric acid for ammonia absorption in the distillation. Phosphate was determined on a separate 0·05 g. of oven-dry material by digestion with sulphuric and nitric acids. The development of colour was carried out by a modification of the method reported by Greenhill and Pollard, measurement being made on a Hilger 'Spekker' absorptiometer using 1-cm. cells and No. 1 red glass filters. A standard curve was constructed, from which the 'unknowns' were calculated.

All other elements were determined by a spectrographic method of analysis. For K, Ca, and Mg, chemical determinations on selected samples were used for calibration of the spectrographic plates which were assessed photometrically. In the case of Na, Mn, Cu, and Fe, visual inspection of the plates showed that there were no large or consistent differences between the treatments, and therefore no photometric determinations were made.

Spectrographic analysis. A cathode layer arc method using lathe-turned carbon electrodes (5-mm. rod, 2·5 cm. long, turned to have a shank 1 cm. long of 3·0 mm. diameter bored 1·5 mm. to a depth of 6 mm.) and a current of 6 amps. The arc was focused on the partially masked colimator in the conventional style and a rotating sector was employed to cut down the total light. It was found that arcing at 6 amps. for 2½ minutes with the sector transmitting ¼ of the total light gave suitable images on the plates (Ilford Special Lantern, normal, contrast, developed 2 minutes at 18° C. in I.D.2).

No internal standard was used as no one element was considered suitable for K, Ca, and Mg at one and the same time. Photometric measurements were made of line strength and adjacent background. The lines used were

K	3446·4 Å.
Ca	3158·9 Å.
Mg	2776·7 Å.

For the routine spectrographic analysis a complete replicate of 'tops', lower stems, or roots was arced in duplicate in the carbon electrodes under identical conditions. After the plate had been processed, four samples forming a suitable concentration range were picked for chemical analysis for K, Ca, and Mg. These same samples were then used as standards for the construction of curves by the photometric measurement of line density.

The spectrographic plates served as permanent records and were later visually examined for differences in Na, Mn, Cu, and Fe.

Chemical determination of Ca, Mg, *and* K. Owing to the small quantities of material available for analysis, the usual macro-techniques were unsuitable. A micro-method modified from the procedures employed by Chapman and Eden was devised and found to give satisfactory results. The reagents used were as follows:

1. Alcoholic ammonium hydroxide: 5 ml. 0·88 ammonia solution diluted with 75 ml. alcohol and adjusted to 100 ml. with water.

2. Reducing agent: 0·5 g. amino naphthol sulphonic acid 1:2:4 added to 27 g. sodium bisulphite in 180 ml. water. 20 per cent. sodium sulphite was added to bring the volume to 200 ml. Filter.
3. Sodium cobaltinitrite: (*a*) 25 g. cobalt nitrate crystals dissolved in 50 ml. water to which 25 ml. acetic acid was added.
   (*b*) 150 g. sodium nitrite dissolved in 250 ml. water.
   (*a*) and (*b*) were mixed and air bubbled through for 24 hours to remove oxides of nitrogen. This was filtered.
4. Standard magnesium solution: 0·959 g. mono potassium phosphate was dissolved to 1 litre. This stock solution was diluted 10 times, when 1 ml. = 50 μg. P_2O_5 = 28 μg. MgO.

The following procedure was adopted:

0·0500 g. of oven-dry sample was ashed at 450° in Pyrex ignition tubes 4 × 1 cm. each provided with small side tube near its base. These were found far superior to small dishes for ashing, baking, and transference. It was convenient to have an asbestos rack upon which these tubes could be rested whilst ashing, &c. The ash was treated with a few drops of HNO_3 and evaporated to dryness on a hot plate, when ashing was repeated. The final residue after treatment with acid and baking was digested with a few drops of 2N HCl on a hot plate and filtration and washing was made into a 10-ml. centrifuge tube using suction. The extract was diluted to approximately 4 ml. and 0·2 g. NH_4Cl added, together with 0·5 ml. 10 per cent. oxalic acid, one drop acetic acid, and two drops bromo cresol green indicator. After standing in a beaker of boiling water for 5 minutes, dilute ammonia ('0·880' diluted 4 times) was added dropwise with stirring until the first shade of emerald green appeared. After a further ½ hour in the beaker, the tubes were cooled and centrifuged at 2,500 r.p.m. for 5 minutes and the supernatant liquid poured off into a 30-ml. beaker, together with four subsequent washings of 2 ml. of water. The residue was dissolved by heating in a water-bath with 1 ml. 10N H_2SO_4 plus a little water. Titration was carried out hot using N/50 $KMnO_4$, when 1 ml. N/50 $KMnO_4$ = 0·00056 g. CaO.

The solution in the 30-ml. beaker was transferred to a 25-ml. graduated flask and diluted to the mark. A 5-ml. aliquot was treated with 0·1 g. citric acid, 1 ml. '0·880' ammonia in a 10-ml. centrifuge tube, and then cooled in ice. To the cold solution 1 ml. of ice-cold 5 per cent. $(NH_4)_2HPO_4$ solution was added and stirred vigorously until precipitation occurred. After standing overnight at about 5° C., the precipitate was centrifuged out at 2,500 r.p.m. and washed three times with dilute ammonia and finally with alcoholic ammonia. 0·5 ml. of 10N H_2SO_4 was added, the precipitate dissolved by immersion for ½ hour in a water-bath. 6·5 ml. of water was added and any undissolved particles centrifuged to the bottom, 2 ml. 2·5 per cent. ammonium molybdate solution added, followed by 0·4 ml. reducing agent. After mixing by inversion and standing 15 minutes the colour was measured in a Hilger Spekker Absorptiometer using 1 cm. cells and No. 1 red glass filters. A standard curve was constructed from which the 'unknowns' were calculated.

A 10-ml. aliquot as above was transferred to a 30-ml. beaker and taken to dryness on a hot plate with a few drops of HNO_3. After ashing at 450° for 10 minutes, 2 ml. of water was added together with 1 drop of acetic acid and the whole warmed. The beaker was carefully 'policemaned' and then rinsed into a 10-ml. centrifuge tube, keeping the volume to approximately 4 ml, when 2·5 ml. of cobaltinitrite reagent was added with careful mixing and allowed to stand for 1 hour. Centrifuging was made at 2,500 r.p.m. for 5 minutes, followed by washing with 2 ml. of a saturated solution of $K_2NaCo(NO_2)_6$ and two lots of 2 ml. 70 per cent. alcohol. After draining, 2 ml. of water was added and solution caused by immersion in boiling water. 0·5 ml. 20 per cent. H_2SO_4 was added and the solution titrated hot with N/50 $KMnO_4$ when 1 ml. N/50 $KMnO_4 = 157\ \mu g.\ K_2O$.

The potassium determinations made by this method were checked against the standard macro-method at frequent intervals. Since the work described here was completed, the modification proposed by Chapman (1947) has been tested and found more satisfactory.

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Multiple-allelomorph Incompatibility of Pollen and Style in the Evolution of the Angiosperms

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I. INTRODUCTION

THE sudden rise of the angiosperms in the Cretaceous period to a position of dominance in the Plant Kingdom, as suggested by fossil evidence, is one of the most remarkable features of plant evolution for which no satisfactory explanation has been offered. Considerable evolutionary significance has been attributed to the closed carpel as the outstanding character which distinguishes angiosperms from gymnosperms. It has been supposed that the closing of the investing carpel has given such added protection to the ovules as would account for the rise of the angiosperms, and only secondary importance has usually been attached to other evolutionary 'advances', such as the aggregation of the stamens and carpels into flowers which are often adapted for insect-pollination, extreme reduction of the male and female gametophytes, double fertilization, and the occurrence of vessels in the xylem. Protection of the ovules is usually considered to be either from desiccation or from injury by animals. However, there is no evidence to suggest that living gymnosperms are greatly handicapped by their lack of protection from these agents. Indeed in most conifers and cycads the ovules are only 'exposed' at the time of pollination, and are well protected by cone-scales at all other times, while in *Ginkgo*, *Podocarpus*, and *Taxus* the ovules are at all times freely exposed, showing that even moderate protection is not always essential for survival. It would thus appear that there is no single morphological character or combination of characters that will account satisfactorily for the remarkable evolutionary success of the angiosperms. Their outstanding

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character is adaptability: they show more diverse life-forms and have invaded more extreme habitats than any other group of vascular plants. Accordingly it is worth-while to consider the factors which may increase the power of adaptation of an organism to its environment.

Fisher (1930) and Muller (1932) have shown that the primary advantage of sexual over asexual reproduction lies in the recombination of hereditary differences, thus giving to sexual species the advantage of responding more rapidly than asexual species to the influence of natural selection and therefore an overwhelming advantage in the evolutionary race. Darlington (1939) and Mather (1940, 1943 *a*) have pointed out that outbreeding is essential for sexual reproduction to show this advantage. However, when a species has become well adapted to a particular environment, outbreeding devices may be discarded, since they are necessarily associated with the bringing together of nuclei from different individuals and this means there will always be a finite probability of sexual reproduction failing altogether. A self-fertile individual always has the advantage that sexual reproduction is independent of other individuals of the species. It is common to find that, within any family or genus of plants, some species possess outbreeding devices while others do not. It is probable that most self-fertile species have been derived from outbred ancestors, since their present adaptation could have been acquired most readily through cross-breeding. Mechanisms which reduce or prevent self-fertilization without greatly restricting the ease of cross-fertilization are therefore likely to have been of particular significance in the early stages of the evolution of a species or a group of species of common descent. The key to the success of the angiosperms may therefore be sought in devices which prevent self-fertilization and promote more efficient cross-fertilization than is possible in the gymnosperms, rather than in structural features which are often of less fundamental evolutionary significance.

II. Comparison of Outbreeding Devices in Gymnosperms and Angiosperms

There appear to be two main outbreeding devices in the gymnosperms, dichogamy and dioecism. By dichogamy, or the separation in the time of maturation of the pollen-sacs and ovules, cross-pollination is favoured but rarely made certain, since the times of maturation usually overlap. If they do not overlap, both cross- and self-pollination may be prevented. By dioecism, or the restriction of the pollen-sacs and ovules to different plants, self-pollination is made impossible, but at a heavy cost, since, if the sexes are distributed equally, any given individual can mate with only half the individuals in the population.

Dichogamy and dioecism also occur in the angiosperms: dichogamy here relates to the times of maturation of the pollen-sacs and stigmas. Numerous structural adaptations which favour cross-pollination occur in entomophilous flowers, but these do not prevent pollination occurring between different flowers on one plant, and so, in general, are merely ‘aids’ to cross-breeding.

Gynodioecious species, with female and hermaphrodite individuals in the population, occur in some families of angiosperms, but are less effectively outbred than dioecious species, since the hermaphrodites are self-fertile (see Mather, 1940; Lewis, 1942). Several other outbreeding devices are found in the angiosperms, based on incompatibility of pollen and style. These are worthy of special attention since no comparable mechanisms are known or indeed appear to be possible in gymnosperms.

Incompatibility is brought about by a physiological reaction between haploid pollen-tubes and diploid style, so that the growth of pollen-tubes of certain physiological constitutions is slowed down and eventually stopped, while others continue to grow normally. Two fundamentally different mechanisms for the genetic control, and hence the inheritance, of incompatibility are known in angiosperms (Mather, 1944). In both mechanisms the physiological constitution of the diploid style is determined by its own genotype, but the physiological constitution of the haploid pollen may be primarily determined either by its own genotype or by the physiological constitution, and hence ultimately the genotype, of the diploid anther in which it was formed. Mather refers to these two types as having haplo-diploid and diplo-diploid control of incompatibility, respectively. The haplo-diploid type appears to have as its primary genetic basis a series of mutiple allelomorphs at one locus, while the diplo-diploid type, which is usually although not necessarily associated with heterostyly, appears to be controlled either by one or by two gene loci, each with one dominant and one recessive allelomorph, and with one dominant gene epistatic to the other when there are two loci. With haplo-diploid control, the number of self-incompatible cross-compatible classes is limited only by the number of allelomorphs in the population at the incompatibility locus, whereas with diplo-diploid control, where there are normally only two allelomorphs per locus, the number of possible classes is determined by the number of loci. Not more than two loci have been recorded, and, accordingly, not more than three self-incompatible cross-compatible classes of individuals are known. The types of incompatibility described by Mather (1944) as having haplo-diploid and diplo-diploid control may be called multiple-allelomorph and two-allelomorph incompatibility, respectively. This nomenclature has the advantage of emphasizing the essential similarity, both in genetic basis and in function, of pollen and style incompatibility in the angiosperms and physiological heterothallism in the fungi, where two-allelomorphic and multiple-allelomorphic types can also be recognized (Whitehouse, 1949*b*).

Two-allelomorph incompatibility, when determined by one locus, gives rise to two self-incompatible cross-compatible classes of individuals in a species. When the essential physiological differentiation of the two classes is also associated with a morphological difference, as in distylic plants, then the two classes may be recognized by their short- and long-styled flowers, respectively. In *Armeria maritima* and many other species of Plumbaginaceae, a dimorphism of pollen, or of stigmas and pollen, is known, associated with an incompatibility analogous to that of distylic plants, but with no macroscopic difference between the two types of flowers (see Baker, 1948). This suggests that the essential feature of the incompatibility mechanism is the physiological and not the morphological differentiation of the two classes of individuals. With incompatibility of the two-allelomorphic type determined by one locus, self-fertilization is prevented, but, as with dioecism, only at the cost of reducing the number of possible mates for a given individual to approximately half the total number of individuals in the population. The system has an advantage over dioecism in that every individual is bisexual, but nevertheless the free transfer of genes through the population is severely restricted. It cannot therefore be supposed that the acquisition of incompatibility determined by two allelomorphs at one locus would give the angiosperms any marked superiority over the gymnosperms.

Two-allelomorph incompatibility determined by two loci gives rise to three self-incompatible cross-compatible classes of individuals. These are familiar in plants with tristylic flowers such as *Lythrum salicaria*, where the three classes of individuals are morphologically distinct. In such plants a physiological device is found which appears to elaborate the incompatibility mechanism without being an essential part of it. Each individual has flowers with anthers at two different levels. The pollen from these stamens is physiologically differentiated so that it is fertile only upon a style which has its stigmatic surface at the corresponding level. That this physiological selectivity associated with the morphological differentiation of the three classes of individuals is not essential for the action of the incompatibility mechanism is suggested by the discovery by Riley (1936) that *Capsella grandiflora* shows an incompatibility of pollen and style essentially similar to that of tristylic plants in its genetic basis, since the behaviour of the pollen is controlled, not by its own genotype, but by the influence of the diploid anther in which it was formed. In this species the three classes of individuals, though physiologically distinct, are morphologically alike.

When two-allelomorph incompatibility is determined by two loci, self-fertilization is prevented with less restriction on cross-fertilization than when it is determined by one locus, since with three classes of individuals, if all are equally frequent in the population (the optimum condition), any given individual can mate with two-thirds of the total number of individuals. Furthermore, unlike a dioecious species, every individual is bisexual and potentially seed-fertile. Two-allelomorph incompatibility with two loci thus provides a more efficient cross-fertilizing device than is known in the gymnosperms, but the gain in cross-breeding efficiency as compared with that of dioecious species is not great. Tristyly appears to be known in only four families of flowering plants—Pontederiaceae, Amaryllidaceae, Oxalidaceae, and Lythraceae. It is of course possible that two-allelomorph incompatibility determined by two loci and without morphological differentiation may be widespread in the angiosperms, but since such investigations as have been made of incompatibility in homostylic plants have revealed only one instance of it (*Capsella grandiflora*), it must be concluded that it is probably of relatively rare occurrence. It is therefore unlikely that the success of the angiosperms as a whole can be attributed to two-allelomorph incompatibility determined by two loci.

Multiple-allelomorph incompatibility is the commonest type of incompatibility found in homostylic flowering plants. As previously stated, it has as its genetic basis a multiple-allelomorphic series at one locus, such that pollen-tubes having an allelomorph in common with the tissue of the style have a reduced growth-rate and fail to reach the ovules. Thus, if the allelomorphs are denoted by S_1, S_2, S_3, &c., then the growth of S_1 and S_2 pollen-tubes is inhibited on an S_1 S_2 style, but that of S_3, S_4, S_5, &c., pollen-tubes is not. The number of allelomorphs at the incompatibility locus in wild or cultivated populations has been found to be large in every species that has been investigated. Thus, East and Yarnell (1929) found 15 different allelomorphs in a sample of 32 allelomorphs (16 plants) of *Nicotiana alata grandiflora* and hybrids of this species with *N. Forgetiana*. Other experimental data by various authors are quoted by Williams (1947). One of the most thorough investigations has been that of Emerson (1940), who found 45 allelomorphs in *Oenothera organensis*, where the total natural population of the species consists of only about 500 individuals. Extensive studies have been made on natural and cultivated populations of species of *Trifolium*, and in every instance a large number of allelomorphs has been found. The published data are presented in Table I. In the last column the estimated total number of allelomorphs in each population is given, based on the assumption that all the allelomorphs are equally frequent in the population. The method given by Whitehouse

TABLE I

Data from Experimental Investigations of the Number of Incompatibility Allelomorphs in Populations of Species of Trifolium

Species.	Author and reference.	Source of plants studied.	No. of individuals in which both alleles were identified.	No. of individuals in which one allele was identified.	Maximum no. of alleles possible in sample.	No. of different alleles recorded.	Estimated total number of alleles in population.
Trifolium pratense	Williams, W. (1947), from data of Williams, R. D. (1939)	Non-pedigree population of 'English late-flowering red clover'.	24	0	48	41	150
		Non-pedigree population of 'English broad red clover'.	18	2	38	34	170
Trifolium repens	Atwood (1942)	Widely separated localities in America, and one plant from New Zealand.	9	1	19	18	175
		Widely separated localities in America.	17	0	34	28	85
	Atwood (1944)	Isolated natural pasture of approximately 1 acre.	0	49	49	36	75
		Natural pasture of nearly 100 acres.	0	49	49	39	100
Trifolium hybridum	Williams, W. (1948)	Canadian commercial seed population.	10	1	21	13	20

(1949*a*) for estimating the number of allelomorphs at the loci for heterothallism in fungi is equally applicable to incompatibility in flowering plants and has been used to obtain these figures. The method of Bateman (1947) gives approximately the same figures. It is evident that the number of allelomorphs at the incompatibility locus in populations of species of *Trifolium* is usually of the order of magnitude of 100. This means that the cross-fertilizing efficiency of these plants will be very near to 100 per cent. In fact, Atwood (1944) has found that there was only about 1 per cent. of cross-incompatibility in natural populations of *Trifolium repens*.

This extraordinarily efficient device for preventing self-fertilization, and yet hardly restricting cross-fertilization, is already known to have a very widespread distribution in the angiosperms. East (1940) records the known distribution of incompatibility in flowering plants, but he does not discriminate between the two-allelomorphic and multiple-allelomorphic types. However, in view of the apparent rarity of two-allelomorph incompatibility in homostylic plants, it appears justifiable to conclude that the great majority of instances of incompatibility that are not associated with any morphological differences between the cross-compatible individuals will prove to be of the multiple-allelomorphic type. East (1940) records the occurrence of incompatibility not associated with heterostyly in over 150 genera of angiosperms distributed through 38 families[1] and 19 orders, and including both Monocotyledons and Dicotyledons. In addition, incompatibility has been reported in *Corylus* (Betulaceae) by Schuster (1924). Since only a very small proportion of the total number of species of angiosperms has been tested experimentally for incompatibility, the true distribution is undoubtedly much wider than the existing data have revealed.

East (1940) has pointed out that incompatibility appears to have played an important part in the evolution of the flowering plants, but he does not attempt to estimate the relative evolutionary importance of the two main types of incompatibility. Mather (1940) considers that the primary advantage of incompatibility over dioecism as an outbreeding device lies in the reduction of gametic loss, since in many incompatible species there are fewer individuals upon which pollen from a given flower fails to function. In a later paper (Mather, 1944) he compares the efficiency of the two main types of incompatibility, and points out that the cross-breeding efficiency is higher and the gametic loss lower with haplo-diploid (multiple-allelomorphic) incompatibility than with the diplo-diploid (two-allelomorphic) type. He also points out that whereas multiple-allelomorph incompatibility requires a minimum of

[1] Gramineae, Bromeliaceae, Commelinaceae, Liliaceae, (Amaryllidaceae), Iridaceae, Zingiberaceae, Orchidaceae, Nyctaginaceae, Nymphaeaceae, Ranunculaceae, Papaveraceae, Cruciferae, Resedaceae, Saxifragaceae, Rosaceae, Leguminosae, Geraniaceae, Euphorbiaceae, Malvaceae, Passifloraceae, Begoniaceae, (Lythraceae), Onagraceae, (Primulaceae), (Plumbaginaceae), Apocynaceae, Asclepiadaceae, (Polemoniaceae), (Boraginaceae), Solanaceae, Scrophulariaceae, Acanthaceae, (Rubiaceae), Caprifoliaceae, Campanulaceae, Goodeniaceae, Compositae. Families enclosed in brackets contain heterostylic species (two-allelomorph incompatibility), and hence incompatibility recorded in the homostylic species of these families is more likely to be of the two-allelomorphic type than it is in the other families.

three allelomorphs for its operation, the two-allelomorphic type requires only two, and consequently the latter type could probably arise in evolution more readily than the former. He concludes that the relative evolutionary advantages of the two types of incompatibility do not differ markedly, the lower cross-breeding efficiency and greater gametic loss of the two-allelomorphic type being compensated by the greater likelihood of its originating by mutation.

The significance of cross-breeding efficiency appears to have been greatly underestimated in these evaluations of the relative importance in evolution of dioecism, two-allelomorph and multiple-allelomorph incompatibility. Since all three of these mechanisms entirely prevent self-fertilization, their outbreeding efficiencies have been regarded as maximal and thus equal. Indeed if outbreeding is measured solely by the degree of heterozygosis in the individuals of the population, then clearly all three methods achieve heterozygosis for every individual, and, if mating is spatially at random, all methods will be equally efficient. However, the cost of achieving such prevention of inbreeding should be measured by the extent to which cross-breeding is also restricted. With dioecism and two-allelomorph incompatibility this cost is heavy, but with multiple-allelomorph incompatibility, if the number of allelomorphs is large, cross-fertilization is almost unrestricted. Consequently, the chance of fertile cross-pollination occurring and of new favourable gene combinations appearing is greater in species with multiple-allelomorph incompatibility than in those with dioecism or two-allelomorph incompatibility. Fisher (1930) has shown that natural selection has little influence in promoting the spread of a favourable mutant gene when that mutation first appears. Thus any other mechanism which favours wide and rapid dispersal of genes through the population of a species will reduce the risk of mutant genes being lost, since so long as a gene is confined to a comparatively few individuals, there is a fairly high probability that these may ultimately leave no descendants. Hence, in general, a favourable mutation will have to appear fewer times in a multiple-allelomorph incompatible species than in a dioecious or two-allelomorph incompatible species, before it is likely to establish itself and spread through the population. Thus, given suitable environmental conditions, species possessing multiple-allelomorph incompatibility will be capable of a higher rate of evolution and have a greater power of adaptation than species without it. Multiple-allelomorph incompatibility therefore appears to be of much greater evolutionary significance than either dioecism or two-allelomorph incompatibility, and may indeed be the key to the success of the angiosperms.

It is significant, moreover, that the analogous phenomenon of multiple-allelomorph heterothallism in the fungi appears to be characteristic of the Hymenomycetes and Gasteromycetes, where it is evidently of extremely widespread occurrence, and may well be the primary cause of the evolutionary success of these Higher Basidiomycetes (Whitehouse, 1949*a*, *b*). A comparison of the efficiency of the main genetic systems known in the Plant Kingdom for restricting or preventing inbreeding, while maintaining a considerable degree of cross-fertility, is shown in Table II. It will be observed that the outbreeding systems known in the fungi find an exact parallel in those known in the angiosperms. However, the outbreeding devices of plants such as the angiosperms with sexual differentiation determined by the diploid phase are more efficient than their counterparts in such plants as the fungi with no sexual differentiation of the diploid phase, since in the latter, unlike the former, self-fertilization of haploids derived from one diploid is never entirely prevented (see Mather, 1942). It is evident from Table II that multiple-allelomorph incompatibility in the angiosperms is an almost perfect outbreeding device, superior to that known in any other group of plants.

TABLE II

Comparison of the Efficiency of the Main Genetic Systems known in the Plant Kingdom for reducing Self-fertility while maintaining Cross-fertility. In the Column headed Self-fertility is given the Mean Percentage Frequency of Fertile Matings between two Haploid Individuals (in A) or two Gametes (in B) chosen at Random from the Progeny of any one Diploid, Dikaryon, or Fertile Haploid-pair. Under Cross-fertility is given the Mean Percentage Frequency of Fertile Matings between Individuals (Haploid in A, Diploid in B) chosen at Random from the General Population of the Species

Outbreeding system.	Genetic basis where known.	Known distribution.	Self-fertility.	Cross-fertility.
A. *Plants with no sexual differentiation in diploid phase.*				
Haplo-dioecism	Pair of differential chromosome segments (one sex with X, the other with Y).	Algae; Fungi; Bryophyta.	50 %	50 %
Two-allelomorph heterothallism.	Pair of allelomorphs at one locus.	Algae; Fungi.	50 %	50 %
Multiple-allelomorph heterothallism.	Multiple allelomorphs at one or two loci.	Fungi (Hymenomycetes and Gasteromycetes).	50 % (one locus) or 25 % (two loci)	Almost 100 %
B. *Plants with sexual differentiation determined by diploid phase.*				
Dioecism.	Pair of differential chromosome segments (one sex XX, the other XY).	Algae (*Fucus*, *Codium*); Gymnosperms; Angiosperms.	0 %	50 %
Two-allelomorph incompatibility.	One or two loci each with a dominant and a recessive allelomorph; when two loci, one dominant gene epistatic to the other.	Angiosperms (Heterostylic species; many Plumbaginaceae; *Capsella grandiflora*).	0 %	50 % (one locus) or 66·7 % (two loci)
Multiple-allelomorph incompatibility.	Multiple allelomorphs at one locus.	Angiosperms.	0 %	Almost 100 %

Accordingly, in the next two sections of this paper an outline will be given of the probable evolutionary consequences of the acquisition by a gymnospermous plant with well-protected ovules, of multiple-allelomorph incompatibility between the pollen and the tissue protecting the ovules. The course of evolution that would be expected to follow is compared with the known facts of the evolution of the angiosperms.

III. PROBABLE EVOLUTIONARY CONSEQUENCES OF THE ACQUISITION BY A GYMNOSPERMOUS PLANT WITH WELL-PROTECTED OVULES OF MULTIPLE-ALLELOMORPH INCOMPATIBILITY BETWEEN THE POLLEN AND THE PROTECTING TISSUE

The available evidence suggests that closure of the carpel was not an easy step in the evolution of the flowering plants, and that it conferred little immediate advantage upon those species which acquired it. Indeed, the germination of pollen-grains upon the 'stigma' of a closed carpel would appear to present greater hazards than germination upon the nucellus of individual ovules, in that the pollen-tubes would be faced with a longer and thus more precarious journey to the embryo-sacs. However, in a plant with well-protected ovules, that is, with a nearly closed carpel, if pollen sometimes germinated upon the carpel tissue instead of at the micropyles of the ovules, and if the pollen-tubes occasionally penetrated the carpel tissue successfully and reached the ovules, then a physiological antagonism developing between the cells of the carpel and pollen-tubes of similar genetic constitution could become highly effective in promoting cross-fertilization. Nevertheless, it appears unlikely that such an antagonism could attain sufficient selective value in a gymnosperm, since the thickness of nucellar tissue which the pollen-tubes must penetrate to reach the embryo-sac is hardly sufficient to be capable of interposing a physiological barrier. In the cycads the nucellus is comparatively thick, but since the pollen-tubes retain their original function of absorption of nourishment and do not act as sperm-carriers, little opportunity would appear to be offered for the operation of an incompatibility mechanism based on the inhibition or retarding of growth of pollen-tubes. Mather (1944) has indicated that incompatibility controlled by multiple allelomorphs is not likely to evolve easily, since a minimum of three allelomorphs is required before the system can begin to operate. However, as soon as an antagonism appears between carpel tissue and pollen tubes of similar constitution, the ovules of a monoecious species would be protected from the individual's own pollen, and circumstances would be favourable for the subsequent development of the highly efficient cross-breeding device of multiple-allelomorph incompatibility.

Such development would depend upon (*a*) the chance occurrence of the necessary three initial allelomorphs at the incompatibility locus, and (*b*) such conditions as a changing environment, under which natural selection would favour the occurrence of the maximum degree of outbreeding with its associated high frequency of recombination. Natural selection would then favour the development of a large number of allelomorphs at the incompatibility locus. It would also favour complete closing of the carpel, thus eliminating the risk of some pollen escaping the incompatibility 'sieve'. Furthermore, natural selection would favour the development and maintenance of a considerable thickness of carpel tissue through which the pollen-tubes must grow. This would afford opportunity for expression of the differential growth-rate between compatibile and incompatible pollen-tubes, which forms the basis of the incompatibility mechanism.

The multiple-allelomorph incompatibility system, when first established, would almost certainly be confined to a single species, since the very improbability of its appearing at all renders highly unlikely the chance of its appearing and becoming established more than once in a short interval of time. This species would have a more efficient outbreeding mechanism than any contemporary species of vascular plants, and would thus be expected to show greater hereditary variability and hence greater plasticity and potential adaptability than other species. In a suitable environment it would be expected to succeed and spread, and through the normal action of isolating mechanisms to become split into distinct species, which in their turn might also evolve new forms and undergo speciation. Such new species would likewise be expected to show greater hereditary variability and hence greater adaptability than their gymnospermous ancestors, both on account of their highly efficient outbreeding mechanism, and also, as Fisher (1930) has shown, because mutant genes can become established more readily in an increasing population than in a fixed one. Adaptability would render these species potentially successful in evolution, and would, in general, favour displacement of many gymnosperms by angiospermous plants. Under such changing conditions, with the dominant plants of the earth being replaced by new species, conditions would be ideal for an efficient outbreeding mechanism to be favoured by natural selection. Conditions would thus be unstable: the greater the success achieved by the angiosperms the greater would be the opportunity of further success.

Thus it seems likely that the first angiosperm with multiple-allelomorph incompatibility might soon give rise to a rapidly diverging and multiplying, and highly successful group of plants. In the evolutionary race the gymnosperm would to a large extent be unable to compete with the angiosperm with multiple-allelomorph incompatibility owing to the greater efficiency of spreading and recombination of genes in the latter, in the same way that asexual organisms would be unable to compete with sexual organisms under changing environmental conditions requiring ready powers of adaptation.

Ultimately the environmental conditions for the individual angiosperm species might be expected to become more stable when the less adaptable species of gymnosperms had been eliminated and the supremacy of the angiosperms had been attained. This relative stabilization of the biotic environment would be expected to slow down the rate of evolution of new forms, for the following reasons: Firstly, the highly efficient outbreeding mechanism would no longer be of such significance, and the disadvantage necessarily associated with any outbreeding device, namely, the risk of fertilization failing altogether, would be likely to cause natural selection to favour the discarding of the incompatibility in many species. Mather (1943 *b*) has pointed out that a multiple-allelomorph incompatibility system can be counteracted, either by the development of a fertility allelomorph at the incompatibility locus, or by the action of modifying genes at other loci. The inbreeding that is likely to follow the development of self-compatibility would be expected to slow down evolutionary change in these species. Secondly, in all species, including those in which multiple-allelomorph incompatibility was retained, the more static environmental conditions would also tend to retard the rate of evolutionary change, since many of the species would have reached an optimum state of adaptation to their particular environment.

It would therefore be expected that, when the angiosperms had become dominant and comparative stability was reached, a marked retardation of the rate of angiosperm evolution might occur. In self-compatible species, if environmental conditions subsequently again favoured outbreeding, the development of such mechanisms as dichogamy, dioecism, and two-allelomorph incompatibility would be more likely than the reappearance of multiple-allelomorph incompatibility, owing to the unlikelihood of the chance appearance by mutation of the necessary minimum of three allelomorphs for the latter mechanism to begin to operate. Those species which retained multiple-allelomorph incompatibility would probably become the chief progenitors of new forms, while those species with less efficient outbreeding devices would be expected to evolve only slowly, and those which were often inbred would be expected to remain relatively unchanged.

IV. Evidence from Present Knowledge of Angiosperm Evolution

The hypothetical course of evolution traced in the previous section shows obvious similarities to the known facts of angiosperm evolution. The following points of resemblance may be noted:

i. A very long interval of time, of the order of magnitude of 200 million years, appears to have elapsed between the rise of the gymnosperms to a position of abundance, which apparently occurred in Devonian or early Carboniferous times, and the rise of the angiosperms apparently in the mid-Cretaceous period. This long interval of time would be explained if, following the adaptation of the pollen-tube as a sperm-carrier, simple closure of the carpel and germination of the pollen upon a stigma was insufficient alone to give angiospermy a selective advantage in evolution, but required, in addition, the chance occurrence of multiple-allelomorph incompatibility.

ii. All known fossils of angiospermous plants appear to be related directly to living angiosperms. This fact supports the view that although a nearly closed ovary may have arisen in several different groups of gymnosperms, a fully closed ovary appears to have been acquired successfully only once.

iii. There appear to be very few living 'angiosperms' in which the carpel is not completely closed, and where such carpels occur, as in *Reseda*, it is likely that the condition is derived and not ancestral. Such semi-angiosperms might have been expected to occur frequently by analogy with the gradations found in gymnosperms between freely exposed ovules as in *Ginkgo*, *Podocarpus*, and *Taxus*, and well-protected ovules as in *Pinus* and most cycads. The rarity of the semi-angiospermous condition and its apparently derived character would be accounted for if, in the early stages of evolution of the angiosperms, selection had favoured complete closure of the carpel, as the present hypothesis presumes, with the result that no semi-angiospermous species survived.

iv. The following facts support the hypothesis that all living angiosperms have been derived from a single ancestral species: (*a*) the uniformity of the male and female gametophytes of the angiosperms; (*b*) the very widespread occurrence of double fertilization within the group; and (*c*) the uniform basic plan of the stamens, comprising two anther-lobes each with two pollen-sacs having a sub-epidermal thickened layer and a common line of dehiscence.

v. The hypothesis that a very rapid evolutionary divergence of the angiosperms occurred in the earliest phase of their existence is supported by the simultaneous appearance in the earliest angiosperm fossils of many diverse types.

vi. The hypothesis that the angiosperms rose abruptly to world dominance is supported by the fact that the earliest plant remains showing undoubted relationship to living angiosperms are a few isolated fossils from the Jurassic period, yet by the Upper Cretaceous period the angiosperms had become dominant on the earth.

vii. Many Cretaceous angiosperm fossils and most of those from the Eocene and later periods can be clearly related to living families of angiosperms (Thomas, 1936, 1947). This is in keeping with the hypothesis that in the later phase of their existence the rate of evolution within the angiosperms has in general been slow.

In addition to the above points of resemblance between the hypothetical course of evolution traced in the previous section and the known facts of angiosperm evolution, certain deductions, which may lie open to confirmation by further observation, can be drawn from the hypothesis.

Firstly, if the hypothesis is valid, it is probable that in the evolution of the various types of ovary, selection would favour the development and maintenance of a considerable thickness of carpel tissue through which the pollen-tubes must grow. Hence a long style would tend to be favoured, perhaps of basal origin, or associated with an inferior ovary. It is also possible that the transmitting tissue (see Thomas, 1934), along which the pollen-tubes pass from stigma to ovule, has evolved in relation to efficient functioning of the incompatibility mechanism. Furthermore, many features of the carpel structure of flowers, such as the so-called 'false-septum' of the Cruciferae and the superposition of the stigmas and ovary partitions in the Papaveraceae, may have evolved in response to the selective advantage of an increased thickness of carpel tissue interposed between stigma and ovule.

Secondly, the frequency and distribution of the occurrence of various outbreeding devices in gymnosperms and angiosperms should provide evidence bearing upon the truth of the hypothesis. This is discussed in the following section.

V. EVIDENCE FROM THE FREQUENCY AND DISTRIBUTION OF THE CHIEF OUTBREEDING DEVICES IN GYMNOSPERMS AND ANGIOSPERMS

The outbreeding devices which will be considered are dioecism, which is found in both gymnosperms and angiosperms, and two-allelomorph and multiple-allelomorph incompatibility, which are known only in angiosperms.

i. *Dioecism*

Among living gymnosperms, which comprise about 600 species, hermaphrodite flowers are unknown except as an abnormality, and all the species can be classified either as monoecious[1] or dioecious. From published data it would appear that approximately 50 per cent. of the species are dioecious, distributed as follows: All the 80 species of Cycadales and the one species of Ginkgoales are dioecious. About one-third of the 450 species of Coniferales appear to be dioecious, including most of the species of *Agathis*, *Araucaria*, *Cephalotaxus*, *Dacrydium*, *Juniperus*, *Podocarpus*, and *Taxus*. Almost all the 70 species of Gnetales appear to be dioecious.

Among fossil gymnosperms, hermaphrodite flowers are known only from certain genera of Bennettitales (Cycadeoidales), such as *Williamsoniella* and *Cycadeoidea*. It may be that the risk of inbreeding was reduced in these plants by dichogamy, perhaps associated with synchronization of the time of flowering of all the flowers on one plant. Throughout all the other groups of fossil gymnosperms, unisexual flowers seem to have prevailed, but whether individual species were monoecious or dioecious it is impossible to say.

In the angiosperms, with some 133,000 species, data concerning the relative frequencies of hermaphrodite, monoecious, and dioecious species are far from complete, but nevertheless are sufficient to show that the proportion of hermaphrodite species is quite different from that existing in the gymnosperms. Thus, Yampolsky, C. and H. (1925), on the basis of available data, found that 70 per cent. of the genera of angiosperms contain only hermaphrodite species, and only 5 per cent. of the remaining genera contain only dioecious species. In the British flora, according to the data of Lewis (1942), the frequencies of hermaphrodite, monoecious, and dioecious species are 92·2, 5·4, and 2·4 per cent. respectively. From information given by Cheeseman (1925), the corresponding figures for the flora of New Zealand appear to be approximately 77·5 per cent. hermaphrodite, 9 per cent. monoecious, and 13·5 per cent. dioecious. Although complete data are not available, there can be no doubt that a majority of the species of angiosperms have hermaphrodite flowers.

One of the outstanding differences between the reproductive structures of gymnosperms and angiosperms is thus the high frequency of occurrence of hermaphrodite flowers in the angiosperms and the rarity of this condition in the gymnosperms. This suggests that the evolution of the hermaphrodite flower of angiosperms was favoured by the occurrence of incompatibility of pollen and style, since, as already indicated, incompatibility appears to be unknown in the gymnosperms. Without incompatibility, species with hermaphrodite flowers are likely in general to be readily self-fertilized, since even when dichogamous the times of maturation of stamens and stigmas may overlap. Moreover, this and other devices which tend to preclude the self-pollination of individual flowers rarely prevent pollination between different flowers on the same plant. It appears from East's (1940) data on the occurrence of incompatibility in angiosperms that hermaphrodite flowers occur in almost all the species in which incompatibility of pollen and style has been demonstrated. This is in keeping with the view that the hermaphrodite flower typical of the angiosperms may have evolved successfully in consequence of the occurrence of incompatibility of pollen and style.

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[1] The term monoecious as used in this paper indicates species in which both sexes are borne on the same plant, but in which the individual flowers are unisexual.

Lewis (1942) has presented evidence for the view that dioecious species of angiosperms have in general been derived from monoecious or hermaphrodite ancestors. This evidence is obtained from the frequency and distribution of dioecism in the group and from genetical studies, and may be summarized as follows:

(*a*) *Systematic evidence.* Dioecism, though comparatively uncommon in angiosperms, is very widely distributed and frequently occurs in isolated species or genera of otherwise monoecious or hermaphrodite families.

(*b*) *Genetical evidence.* Darlington (1939) has shown that a series of stages can be traced in the degree of elaboration of the sex-determining mechanism in dioecious organisms. These steps are the following: (1) Unisexual individuals originate from bisexual by the action of one or two genes. (2) A structural chromosomal change occurs in the region of the sex chromosome which carries the sex genes, such that crossing-over is suppressed within this region. Differentiation of the sexes can then proceed, since genes determining secondary sexual characters remain permanently linked to the sex appropriate to them. One sex then has two X-chromosomes and the other one X- and one Y-chromosome. (3) Since the Y-chromosome never pairs with the Y-chromosome of other individuals of the species, the differential segment of this chromosome never undergoes crossing-over, and recombination of genes within this segment is virtually impossible. This is disadvantageous to the species, and in time the genes of the Y-chromosome are replaced by genes on the other chromosomes (autosomes), the Y-chromosome becoming inert. Sex determination is then dependent on the proportion of X-chromosomes to autosomes.

The majority of dioecious angiosperms that have been investigated possess a sex-determining mechanism which is at a comparatively early stage in this process of elaboration. This suggests that dioecism is of comparatively recent origin in these species (Lewis, 1942).

The conclusions, (*a*) that the evolution of the hermaphrodite flower of the angiosperms may be related to the occurrence of incompatibility, and (*b*) that dioecious angiosperms are mostly of comparatively recent evolutionary origin from monoecious or hermaphrodite types, support the present hypothesis.

ii. *Two-allelomorph incompatibility*

Apart from its occurrence in *Armeria maritima* and other members of the Plumbaginaceae with dimorphic pollen or stigmas, and in *Capsella grandiflora*, the known distribution of two-allelomorph incompatibility is represented by the distribution of heterostyly. East (1940) records the existence of heterostyly in 17 families[1] of angiosperms distributed through 11 orders, with a possibility of its occurrence in 3 other families.[2] In many of these families the heterostylic species are confined to a few genera, or even to part of one genus, as in *Linum*. Lewis (1943 *a*, *b*, 1944) has shown that the physiological mechanism of incompatibility in *Linum grandiflorum* is based on differences of osmotic pressure, whereas in *Primula obconica* and *P. sinensis* it is analogous to the mechanism of multiple-allelomorph incompatibility and probably dependent on a specific reaction between protein molecules. The scattered distribution of heterostyly and the variable physiological mechanism of the incompatibility associated with it, suggest that this type of incompatibility has evolved comparatively recently and independently in a number of widely separated families. This is in keeping with the present hypothesis. In the Rubiaceae, where a large number of genera show heterostyly, and in the Plumbaginaceae (see Baker, 1948), it appears likely that two-allelomorph incompatibility evolved comparatively early in the evolutionary history of each family.

iii. *Multiple-allelomorph incompatibility*

Data are at present inadequate for any generalization to be made in relation to the distribution of multiple-allelomorph incompatibility in the angiosperms. As already indicated, there are many species in which it is not known whether incompatibility is of the two-allelomorphic or multiple-allelomorphic type, though it is probable that multiple-allelomorph incompatibility occurs in the majority, on account of the much greater frequency of this type among the homostylic angiosperms which have been investigated. East (1940) records the highest frequency of occurrence of incompatibility not associated with heterostyly in the families Gramineae, Liliaceae, Papaveraceae, Cruciferae, Rosaceae, Solanaceae, Scrophulariaceae, and Compositae. In these families there are indications that probably 10–25 per cent. of the species show incompatibility. However, little significance can be attached to this list, since in a great many other families few or none of the species have been investigated. It cannot yet be stated categorically that there is no family in which multiple-allelomorph incompatibility does not occur, since all the species of one family have not yet been tested. However, it is probable that multiple-allelomorph incompatibility is absent in families composed entirely of dioecious species, since dioecism and incompatibility appear to have the same function of promoting outbreeding.

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[1] Pontederiaceae, Amaryllidaceae, Polygonaceae, Oxalidaceae, Linaceae, Erythroxylaceae, Hypericineae, Dipterocarpaceae, Turneraceae, Lythraceae, Primulaceae, Plumbaginaceae, Oleaceae, Gentianaceae, Polemoniaceae, Boraginaceae, Rubiaceae.

[2] Amarantaceae, Thymelaeaceae, Verbenaceae.

The fact that the frequency of occurrence of multiple-allelomorph incompatibility is comparatively low in living angiosperms is not at variance with the hypothesis that all angiosperms are descended from a species with this type of incompatibility, for the following reasons:

i. All efficient cross-fertilizing devices are automatically associated with a disadvantage in that there is a risk of failure to set seed, through lack of fertile pollination. Hence under stable environmental conditions the occurrence of a considerable proportion of self-fertile species would be expected (see also Mather, 1940).

ii. It appears that multiple-allelomorph incompatibility can be lost in evolution much more readily than it can be acquired, since, as already stated, it can be counteracted either by a self-fertility allelomorph or by modifying factors at other loci (see Mather, 1943 *b*).

iii. A considerable proportion of living species of angiosperms appear to be polyploid, and it appears that the multiple-allelomorph incompatibility mechanism frequently breaks down in polyploids (see Lewis and Modlibowska, 1942; Lewis, 1944).

It might be argued that the wide distribution of multiple-allelomorph incompatibility among the angiosperms implies an origin analogous to that already attributed to dioecism, namely a comparatively recent and independent development in a large number of different families. However, dioecism can originate by the chance appearance of two mutations in different individuals, one suppressing the male and the other the female organs (Lewis, 1942), while the evolution of multiple-allelomorph incompatibility is likely to occur extremely rarely, since, as already stated, it requires the chance appearance of at least three allelomorphs controlling pollen-tube growth before it can begin to operate (Mather, 1944).

From his survey of the distribution of incompatibility in the angiosperms, East (1940) has concluded that incompatibility is more frequent in herbaceous than in woody families and genera, even allowing for the fact that a larger proportion of herbaceous than woody species has been investigated experimentally. He argued from this that as the woody types were generally thought to be the older in evolution, incompatibility had evidently arisen comparatively recently in many different lines of herbaceous plants. However, if the original angiosperms had all possessed multiple-allelomorph incompatibility, those species which subsequently lost it would be expected to evolve more slowly and hence to show a greater range of ancestral characters than those species which retained it. Thus the woody character, if ancestral, may have remained in those species which lost their incompatibility mechanism, and conversely, the herbaceous 'modern' families may be of comparatively recent origin because they have retained this mechanism. East's findings are therefore not necessarily at variance with the present hypothesis.

The idea that multiple-allelomorph incompatible species occupy the vanguard of evolution in the angiosperms, followed by a trail of less efficiently outbred species evolving more slowly, and with inbred species almost static in the rear, can of course only be applied broadly, since in individual instances many other factors will interact to modify the rate of evolution. It does suggest, however, that when the data concerning the distribution of multiple-allelomorph incompatibility in the angiosperms are more complete, they may provide evidence of the trend of evolution of different groups within the angiosperms.

When sexual reproduction first evolved, it must have caused a complete transformation of the dominant living organisms of the earth, owing to the inability of asexual species to compete with sexual species. All subsequent abrupt increases in the efficiency of sexual reproduction as a means for recombining hereditary differences are likely to have been followed by similar widespread changes, though probably on a smaller scale than that which must have followed the evolution of sexual reproduction itself. The evidence presented in this paper supports the conclusion that the abrupt rise of the angiosperms from the gymnosperms may have been the result of such a transformation, due to the increased efficiency of sexual reproduction made possible by multiple-allelomorph incompatibility.

VI. Summary

It is suggested that multiple-allelomorph incompatibility of pollen and carpel tissue has been the primary cause of the evolution of the closed carpel and of the success of the angiosperms over their gymnospermous ancestors. The profound significance of the closed carpel for angiosperm evolution would then lie in the protection of the ovules, not from desiccation or the attacks of animals, but from fertilization by the individual's own pollen, without appreciably restricting cross-fertilization. This hypothesis is supported by the wide distribution of multiple-allelomorph incompatibility in living angiosperms, and it appears to account for many of the known facts of angiosperm evolution and for their outstanding quality, adaptability.

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The Symbiotic Seed-borne Fungus in the Cistaceae

I. Distribution and Function of the Fungus in the Seeding and in the Tissues of the Mature Plant[1]

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With Plate VII and six Figures in the Text

Introduction

INVESTIGATIONS on the structure and life-history of those fungi which are found in the testa or in the seed tissues of the angiosperms, and the effect of their presence on the seed and its consequent germination, have been carried out by numerous workers. Parasitic fungi frequently contaminate seeds, and the danger in infection of this kind is the carrying over of the fungus from the seed to the next generation (Orton, 1924, 1931; Doyer, 1948).

The majority of fungi found in or on the tissues of seeds are parasites. However, some seed-borne symbiotic fungi have been recorded. Here a symbiotic fungus which penetrates through the tissues of the parent plant enters the seed and is then transmitted through the seed to the daughter plant, with the result that the symbiotic association is set up once more in the new generation. Here again, therefore, the association is a hereditary one.

Rayner (1913, 1915) recorded the presence of a symbiotic fungus which penetrated throughout the tissues of the plant, and into the seeds of *Calluna vulgaris*, and also in other members of the Ericaceae. A similar condition is known in *Lolium* and was described in great detail by Freeman (1903), though here the connexion between the fungus in the root and that in the seed has not been fully elucidated. The degree of penetration of the fungus in *Calluna* and *Lolium* is different, however. In the former it does not penetrate beyond the testa, but in *Lolium* it enters the embryo and penetrates the growing point, often approaching to within two cells of the tip. Bose (1943, 1946) records the presence of a species of *Phoma* in *Casuarina equisetifolia* which penetrates through the cells of the root, stem, and flower. It occurs in the pericarp wing of the fruits and in the seed-coats. Here, as in *Calluna*, the fungus infects the radicle as it emerges on germination. Thus, Bose states, 'every plant is a dual organism, harbouring a fungus in its seed, so that the symbiotic relationship becomes hereditary'.

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[1] Part of a thesis approved for the degree of Doctor of Philosophy in the University of Wales.

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In all these cases when the seed is dormant there is a corresponding dormancy on the part of the fungus. Then the seed germinates, and the growth of the seedling is accompanied by a corresponding renewal of activity of the fungal mycelium, which results in the regular infection of the tissues of the seedling. The fungus passes from plant to plant through succeeding generations. It appears that this method for the propagation of the species and the setting up of a new symbiotic relationship is as successful as the spore method of reproduction, with the consequent renewal of infection of each plant by the fungal mycelium.

The higher plant too seems to depend on the presence of the fungus to complete its reproductive cycle. Rayner (1915) stated that germination and development in *Calluna* are bound up with the infection of the seedling by the fungus from the testa. Freeman (1903) showed that the grains of *Lolium* are incompletely developed in the absence of the fungus. De Jongh (1938) investigated the bacterial symbiont in the seeds of *Ardisia crispa*, and found that the presence of the bacterium in the seed was an essential factor for successful germination. He used the term 'cyclical symbiosis' to illustrate the relationship between the seed-borne symbiont and the plant.

The term 'mycorrhiza' first used by Frank in 1885 means fungus root, and should only be applied to those plants which contain a fungus of a non-parasitic nature in the roots. The term, however, is often loosely used and covers the association between higher plant and fungus, where the fungus spreads beyond the root, into the stem and floral organs and eventually into the seed, from where it reinfects the germinating seedling. To this type of association de Jongh's term *cyclical symbiosis* will be used.

The members of the family Cistaceae, like the Ericaceae, seem to be characterized by the presence of a fungus in the seed-coat. It was known that *Helianthemum chamaecistus* also possessed an endophytic fungus in the roots, and it was thought that the fungus might play a part in the life-cycle similar to that seen in the Ericaceae. Is this seed-fungus a cyclical symbiont? Is the relationship a hereditary one, and is the plant, as Bose suggests in *Casuarina*, a 'dual organism'?

MATERIAL AND METHODS

The greater part of this investigation on these seed-borne fungi in the Cistaceae was carried out on *Helianthemum chamaecistus* Mill. This plant is a calcicole, and occurs frequently on warm dry chalky soils in the south and south-west of England. Plants were examined for the presence of the fungus in the seed and in the root, and in all cases it was found to be present in both these regions. Material was examined while fresh or fixed with formalin-acetic-alcohol (Johansen, 1940). From the outset the greatest difficulty was experienced in obtaining a staining technique which would adequately differentiate the fungal hyphae from the cell contents. A great amount of tannin is present, and this makes it difficult to see the fungus as these tannin deposits tend to pick up the fungal stains. The most successful stain combination was found to be thionin made up in aqueous phenol, with Orange G as a counterstain (Stoughton, 1930). This results in the fungal cytoplasm appearing purple, while the cell cytoplasm stains blue. Fresh material was stained by the lacto-phenol cotton-blue technique.

The distribution of tannin was followed by staining fresh material with 10 per cent. ferric chloride, or by fixing in an aqueous solution containing 5 per cent. formalin and 10 per cent. ferrous sulphate for 24 to 48 hours; this fixed material was washed, dehydrated, and sectioned but not stained as the iron compound both fixes and stains the tannin.

Seedling tissues were examined by the 'squash' method, and were stained with cotton-blue, or with a modification of the iron-alum-haemotoxylin method suggested by Cook (McLean and Cook, 1941).

DISTRIBUTION OF THE FUNGUS IN THE MATURE PLANT

When the dry seed is placed in water a thin transparent coat is seen surrounding the dark brown testa. This coat adheres to the testa in the dry state, and therefore cannot be observed, but it swells up immediately on contact with water. Examination shows that it is made up of large cells containing numerous starch grains. The interesting feature of this coat, however, is the fact that it is permeated by a septate and branching fungal mycelium (Pl. VII, Fig. 1). Since the fungus is found in the outer coat of the seed, the root of the germinating seedling will, in all probability, become infected with the mycelium as, or soon after, it emerges. The adult plant which showed this infection in the roots did not appear to be diseased in any way, and it was thought, therefore, that the fungus might be symbiotic. Does the fungus in the root bear any connexion with the one found in the seed? If it is the same one, does it enter the seedling on germination and penetrate through the plant, entering the growing points and finally the floral organs and the seed itself? Is this in fact a cyclical fungus which passes the whole of its life in the plant and is transmitted from one generation to another through the seed?

The term 'host' will be used to describe the higher plant, and the fungus will be said to 'invade' or 'infect' the cells. These terms do not imply that the fungus is in any way considered as a parasite.

The distribution of the fungus was followed in the root, stem, and leaf of the plant, in the vegetative and floral meristems and in the flower itself. Any changes in host and fungal tissues were noted, together with any variations in intensity and in the form of the fungus throughout the year.

Root

The distribution and general appearance of the mycelium in the root tissues were examined at all periods of the year. In general the roots of *H. chamaecistus* are long and have very few branches. When the lateral branches begin to develop and break through the parent cortex in March or April, two distinct types of branch root can be seen. Some are long and slender, while others are extremely short and tuberous. To the former the term *long root* will be given, and to the latter *tuberous root.* It is usual to find a number of these tuberous roots developing together; then there may be an expanse of root which bears only long roots.

Tubercles similar to those seen in *H. chamaecistus* have been recorded by Rivett (1924) in *Arbutus unedo.* She states that these root tubercles are arrested secondary and successive laterals of the season's growth, and she shows that the arrest of growth is due to the invasion of the tissues by the fungus from the main root.

It is best to carry out a preliminary study of the distribution of the fungus in the roots in the summer months. In the 1-year-old long root, septate branching fungal hyphae can be seen permeating the cortical cells. They enter and leave the cells at the corners. At this period the great majority of the hyphae are found in the cells, and not in the intercellular spaces. The hyphae appear to be normal and show no signs of any digestion, as is so often the case in the endophytic fungi. The infection seems to be much denser in certain regions of the cortex than in others, but this difference in the density of infection in various regions seems to be quite haphazard. McLennan (1926) states that in *Lolium*, also, infection is not universal for each individual root; some are devoid of the fungus for their entire length, while others show infected patches alternating with uninfected regions. The endophyte of *H. chamaecistus* is most prolific in the middle layers of the cortex, and found very infrequently in the outer layers or in the epidermis. Penetration into the stele appears to be prevented by the thick deposit of tannin-like substances in the cells of the innermost layer of cortex and in the endodermis. McDougal and Dufrenoy (1944) showed that the distribution of the endophyte in the cortex of pines does not extend beyond the endodermis in which catechol tannins are formed. Bose (1943) demonstrated that there was a close connexion between the presence of tannin and the activity and spread of the endophytic fungus in *Casuarina*. In *H. chamaecistus* the endophyte, however, has been observed in the vascular tissues. Actual penetration of the fungus from the cortex, through the tannin-filled endodermal cells into the stele has not been seen, and it is thought that these hyphae seen in the vascular parenchyma, and sometimes in the xylem and phloem cells, have penetrated here by an upward growth from the vascular regions of the young root in the previous year before there was much deposition of tannin in the cells.

It is at this period, when the fungal mycelium is in an active state in the cortical cells of the 1-year-old long root, that branch roots arise. As these roots pass out they may or may not become infected with mycelium from the parent cortex; this obviously depends on whether the fungus is present in those regions in which the root arises. If it is present, it surrounds and eventually penetrates into the cells of the young root before they break through the epidermis. In many cases, however, there is no fungal mycelium in this region, and the young root does not become infected. This uninfected branch root breaks through the parent cortex, and growth is normal and a *long root* is formed. This does not mean that this root remains permanently uninfected. As the fungus increases its activity during the summer months, this long root will eventually become infected by branches of the fungal mycelium from the parent root which will penetrate easily and rapidly through the mature, vacuolated cells. The branch root which is surrounded by fungal hyphae in the parent cortex, and is infected before it reaches the exterior, is destined to become a *tuberous root*. The cells increase greatly in size, and except for a small number of cells near the tip, they appear to lose altogether their meristematic activity. This means that there is very little increase in length, but there is a considerable swelling of the root as a result of the increase in size of the individual cells. These grow until they are about 40 μ across, whereas a normal long root cell at the same stage in development is only 16 μ across.

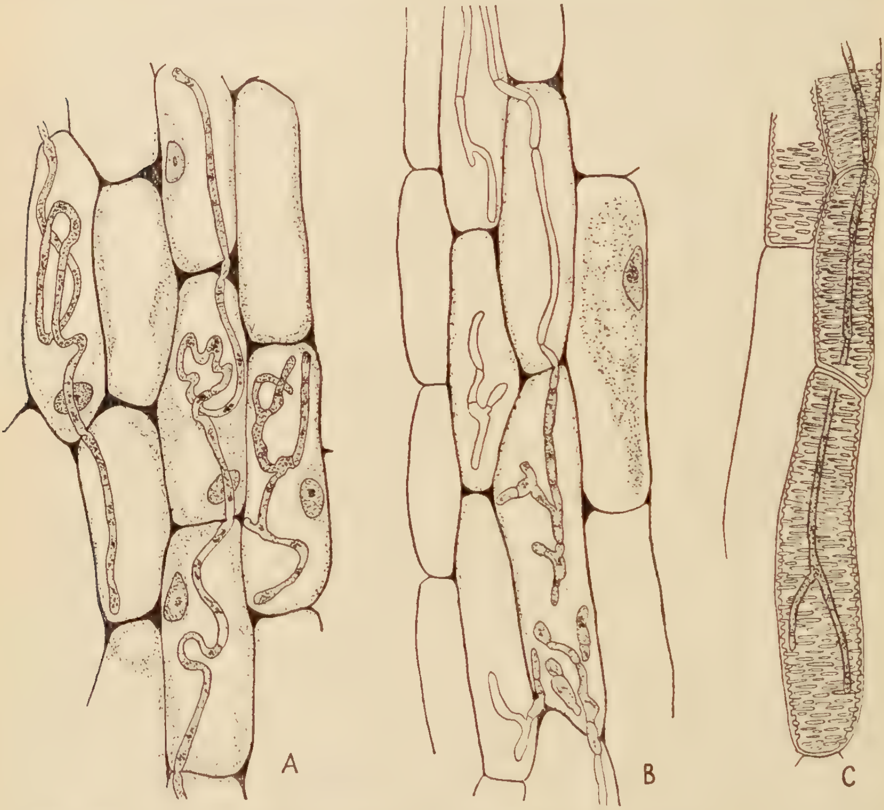
Rivett states that in *Arbutus unedo* tuberization is the result of the successful early establishment of the ectotrophic condition which prevents the continuance of normal root growth. She says that normal laterals show only a few hyphae applied to their surface as they break through the cortex: these roots are the 'straight, long-growing variety' and they continue their growth. Where the hyphae are abundant they invest the root and prevent growth, with the resulting formation of tubercles. In *H. chamaecistus* the formation of long or tuberous roots is not dependent on the density of ectotrophic growth, but is the result of the density of *endotrophic* growth in the parent root at the point at which the branch arises.

It appears that the presence of the fungi in the cortex around the young root, and the consequent infection of the cells at this very early stage, in some way disturbs the metabolism of these cells. They cease to divide, but still increase in size. The increase in size of the cells may be the result of a secretion of certain substances from the hyphae in the parent cortex at the point at which the root arises. It appears that very young cells are incapable of setting up a balanced symbiotic association and that the secretions from the hyphae may disturb the normal activities of the cell. It is rarely that we find mature cells (in which alone we find active fungal mycelium) side by side with meristematic cells; there is usually a gradation from one to the other. It is rarely, therefore, that active mycelium in mature cells will be in contact with meristematic cells. One case of this, however, is seen in the formation of branch roots. That fungal hyphae do upset the normal activities of a meristematic cell is deduced from the fact that branch roots grow normally if there are no hyphae in the cortex around them. The increase in size of the cells, and the consequent tuberization of the root, reminds one of the effect of various growth substances on the cells of roots in particular.

DISTRIBUTION AND CONDITION OF THE FUNGUS IN THE ROOT AT ALL PERIODS OF THE YEAR

During the summer months the mycelium appears to grow very rapidly in the 1-year-old long roots, and extends into the new laterals. During July and August numerous changes take place in the form of the hyphae in the plant, and more particularly in the balance between fungus and host.

The hyphae during this time are extremely vacuolate and have few cross-walls; they branch extensively and form coils in the cortical cells (Text-fig. 1, A). This coiling (Pl. VII, Fig. 2) appears to be followed by the digestion of the hyphae in the cells. The *digestive* process occurs mainly in the 1-year-old long roots and in the tuberous roots. There is very little digestion in the new long roots. Digestion in *Helianthemum* does not seem to be comparable to the clotting and disintegration of the endophyte which is recorded in other endophytic fungi (Rayner, 1915). The hyphae increase in width until they are about twice their normal size, and the hyphal membrane appears to become much thinner. A diffusion of soluble materials may occur from the hypha, through the hyphal membrane, into the cavity of the cell. During the autumn the hyphae in the root are devoid of contents. By this time too the mycelium has broken up into small pieces in each cell, and it is difficult to find the connecting links from cell to cell. During the winter months it is often extremely difficult to find any trace of active fungal mycelium in the cortex



TEXT-FIG. 1. Fungal hyphae in cells of root of *Helianthemum chamaecistus*. A. Coiling and branching of the hyphae in the cortical cells in May. B. Fungus in cortical cells of the long root at the end of the first season's growth. C. Fungal hyphae in xylem element in winter.

of the 1-year-old long root, and the swollen empty hyphae appear to have been completely digested by the host cell. The mycelium, however, appears to be almost unchanged in the current year's long root (Text-fig. 1, B), and it is from here that the new roots will be infected as they arise in the spring and summer of the following year. The mycelium in the intercellular spaces of the cortex of the long root is not digested, but remains in the active hyphal state. This is also the case in the vascular tissues, where septate and branching fungal hyphae can be seen in the xylem elements during the winter months (Text-fig. 1, C).

In the tuberous roots many changes take place during the late summer and autumn. The mycelium, which was mainly intracellular, tends to become more and more intercellular especially at the tip of the root, so that one sees two fairly clearly defined regions. The first is the region with great intracellular infection nearest the main root, and the other is a region of much intercellular mycelium and only slight intracellular infection near the tip. The digestive process is much more drastic in the tuberous roots, and the intracellular hyphae break up into numerous small portions, swell up, and lose their contents. The intercellular hyphae are not affected by this process and the tendency is for the mycelium to grow out through the intercellular spaces of the cortex in between the epidermal cells and thus form a mat of ectotrophic growth on the surface. At the tip of the root, where there is much intercellular mycelium, there is a rapid growth of fungal hyphae towards the exterior, where they branch and form a thick dense mat on the outside of the root. The hyphae here are so interwoven that they give rise to a pseudoparenchymatous layer outside the cortex and epidermis. There are now two even more distinct regions in the tuberous root (Text-fig. 2):

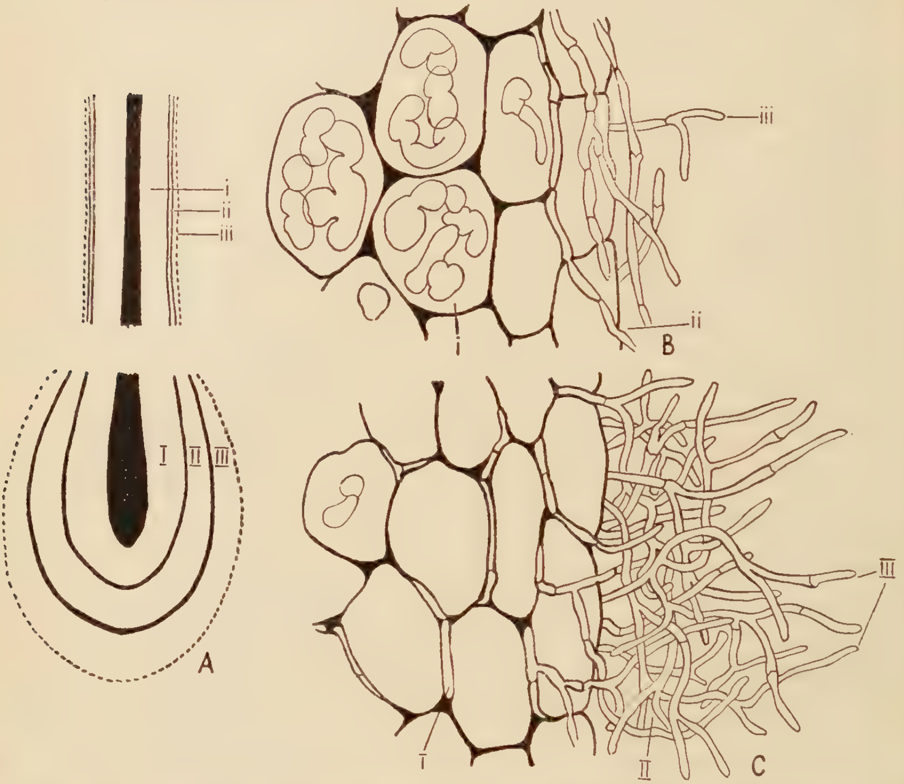
(*a*) Near the main root there is a region of great intracellular infection coupled with only slight ectotrophic growth. The hyphae in the cortical cells have become greatly swollen, they have a very thin membrane and no contents (Text-fig. 2, B).

(*b*) Towards the tip there is a region of great intercellular infection coupled with dense ectotrophic growth. There is only slight intracellular mycelium, and this is in the characteristic digested state. The intercellular mycelium, however, is still in the hyphal state, is full of cytoplasm, and possesses extremely thick walls. It passes out between the epidermal cells and forms the dense mat of interwoven hyphae of a pseudoparenchymatous nature on the surface. From this strands of hyphae pass outward into the soil (Text-fig. 2, C).

It is easy to pick out these tuberous roots in September by their swollen appearance and dark colour: the ectotrophic hyphae can be seen quite clearly with a hand lens. During the winter months the ectotrophic growth is cut off, firstly by the production of a thick layer of cork on the outside of the tuberous root, and secondly by a zone of tannin-filled cells inside this.

The mycelial cycle in the roots can be summarized in the following way:

1. During the spring there is an active fungal mycelium in the 1-year-old long roots. Branch roots arise and develop into tuberous or long roots, depending on whether they become infected or not as they pass through the parent cortex.



Text-fig. 2. Diagram to show type of infection in tuberous root in September in *Helianthemum chamaecistus*. A. L.S. of tuberous root showing the two main regions. B. Region with great intracellular and slight intercellular growth. C. Region with intercellular infection and dense ectotrophic growth: i. cortical cells full of swollen, empty distorted hyphae; I. cortex with unaltered intercellular mycelium; ii. thin mat of interwoven hyphae; II. dense mat of interwoven hyphae forming pseudoparenchymatous layer on the surface; iii. extremely sparse ectotrophic growth; III. loose web of innumerable ectotrophic hyphae.

2. During the summer the long roots increase in size and produce more laterals. The fungal hyphae in the parent cortex are active and they eventually penetrate through the cells of the new long roots.
3. Digestion, preceded by coiling and branching of the hyphae, occurs in late summer in the 1-year-old long roots, and in the tuberous roots. It does not occur in the current season's long roots. The hyphae at the tip of the tuberous root pass into the intercellular spaces and form a dense ectotrophic growth on the surface.

4. In the winter there is very little sign of active mycelium in the old roots but it is still found in the young long roots. The ectotrophic hyphae of the tuberous root are cut off by a layer of cork and tannin cells.
5. In the following spring the now 1-year-old long roots with active mycelium act as a source of infection for active endophytic growth in the newly formed roots.

Stem

It is extremely difficult to find any trace of fungal infection in the stem. The outer and inner cortical cells contain heavy deposits of tannin-like substances, but the middle layers of cortex are devoid of them. The pith also contains a high percentage of tannin-filled cells. These deposits take up the normal fungal stains, and for some time the tannin deposits were taken for digested masses of fungal hyphae in the cells. The branching Holmgren canals are very similar to the masses of partly digested hyphae seen in endophytic mycorrhizal infections.

It is thought that fungal hyphae penetrate through the young plant from the root upwards through the vascular parenchyma of the stem, in and between the cells of the cortex, and sometimes in the xylem and phloem elements. As the cells mature it is difficult to find any mycelium in the stem tissues, but inter- and intracellular mycelium has been observed in the cortex and pith. Active fungal hyphae can also be seen in some of the phloem cells, and in the vascular parenchyma just behind the apical meristem. It seems that the fungus keeps pace with the growth of the apex and enters new tissues as they arise. Fungal hyphae extend upwards in the vegetative apical meristem as far as the fifth and fourth pair of leaf-bases, and from here they extend into the leaves themselves. The fungus does not penetrate into the meristematic cells, and the endophyte was only found in those cells in which vacuolation had commenced. The fungus is most dense in the vascular region at the node. When the axillary buds develop the fungus spreads into the new tissues, and in the small axillary shoot the condition seen in the apical meristem is repeated.

Active fungal growth only occurs in the younger regions of shoot and root, while in the older regions it appears to be absent. There is no apparent connexion between the mycelium in the root and shoot of the adult plant, although shoot infection originally arose from the mycelium in the root. This is probably the result of the digestion and disappearance of the fungi of the older regions of the root so that the connexion between root and shoot is severed.

Leaf

The fungus can be seen in the leaf in the vascular parenchyma in a greatly attenuated form, and it extends also into some of the mesophyll cells. The fungus was never observed in the palisade tissue.

Floral organs

The fungus in the vascular parenchyma extends to the base of the floral meristem. The rudiments of the flower are completely laid down before the fungus penetrates into the receptacular tissue which has now become vacuolate.

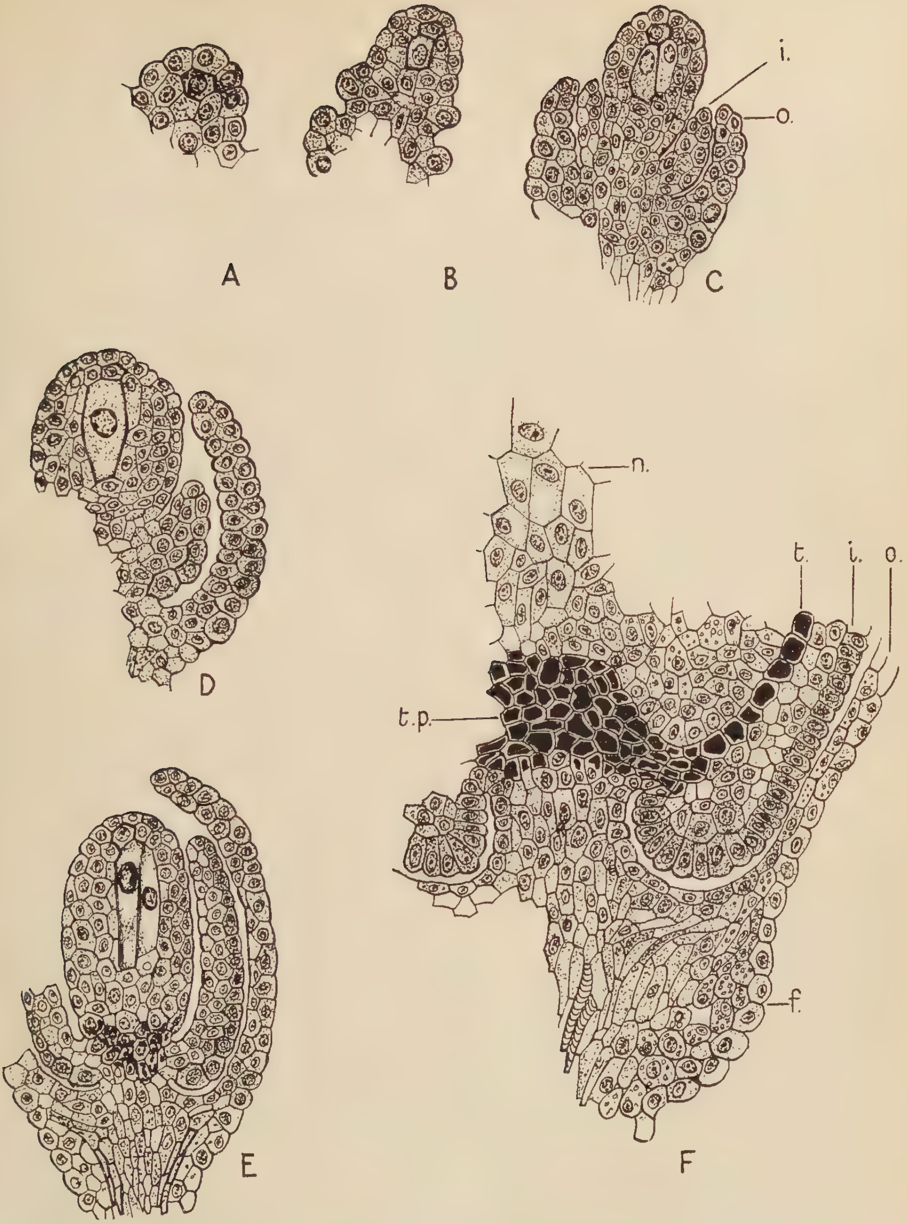
INFECTION OF THE SEED BY THE FUNGUS

The floral bud begins its development as a cone-shaped mass of tissue on the floral apex. These cells are full of cytoplasm and there is no sign of fungal infection. Five sepal and five petal primordia arise on this cone, followed by four tiers of stamen primordia and finally the carpel initials. In the first stages the carpels are crescent-shaped primordia with the concave sides facing the apical meristem of the receptacle. As the growth of the carpels proceeds, lateral veins from the midrib extend in all directions to ramify within the tissues of the carpellary wall, thus laying down the means of supplying the ovule, and later the seed, with nutritive materials, and in this case acting as a means whereby the fungal mycelium may pass rapidly into all parts of the ovary. The ground tissue of the carpel shows an intercellular system of air spaces, and the cells often contain large crystals of calcium oxalate.

Development of ovule

A small protuberance of meristematic cells is formed on the placenta. Integuments arise as rings of tissue around this ovule primordium (Text-fig. 3, A, B, C). Inner and outer integuments develop almost simultaneously, but the latter integument grows more rapidly than the former. It is made up of two layers of cells, while the inner integument is three-layered. These cells of the outer integument grow rapidly; development of the inner integument is slower and very complex. Some interesting and characteristic features occur at the chalazal end of this orthotropous ovule. The basal cells of the outer layer of the inner integument increase in size and multiply rapidly, but instead of producing an upward growth from chalaza to micropyle as is normally seen, these rapidly formed cells produce a bulge of tissue in this region (Text-fig. 3, D, E). This bulge is produced so rapidly and is so great that there is an invagination of tissue, the outer cells being pushed inwards and upwards into the nucellar tissue, at the base of the ovule (Text-figs. 3, F; 4). Thus a ring of invaginated, extremely large columnar cells is formed in this region. These cells do not completely block the entrance from the funicle to the nucellar tissue; they leave a column of elongated undifferentiated parenchymatous tissue, about five cells across in between. This development of the outer layer of cells of the inner integument is a characteristic feature of the ovule of *H. chamaecistus*. The layer is easily recognized by its closely packed, radially placed, thick-walled columnar cells. The inner two layers of cells of this integument remain unmodified, as do the cells of the outer integument.

When the rudiments of the flower have been laid down, rapid growth of the filaments of the stamens and of the petals occurs and the flower opens.



Text-fig. 3. Development of ovule of *Helianthemum chamaecistus*. A. Ovule primordium on placenta. B. Inner and outer integument initials. C and D. Development of the three-layered inner and the two-layered outer integument. E. Development of 'bulge' at the base of inner integument. F. Base of developing ovule showing tannin-filled cells. *i.* inner integument; *o.* outer integument; *t.* tannin layer; *t.p.* tannin plug; *n.* nucellus; *f.* funicle.

After this, all growth is the result of increase in cell size, accompanied by vacuole formation. It appears that this is the stage when the fungus enters the flower parts, and it can therefore play no part in the nutrition of the developing flower.

Penetration of the fungus into the seed

The fungus appears to penetrate rapidly through the vacuolated parenchymatous cells and through the elongated cells associated with the vascular tissue. It passes into the vascular parenchyma of the receptacle, and from here into the ovary wall. If strips of the inner epidermis of the ovary are examined the fungus will be seen penetrating through the cells. It passes from the carpellary tissue, through the placenta to the funicle of the ovule, where it is found in the vascular parenchyma, the cortex, and occasionally in the xylem elements.

If the fungus can penetrate as far as the funicle it should be able to pass from here into the ovule. If the fungus can enter the ovule, and therefore the seed, its entry into the next generation is certain.

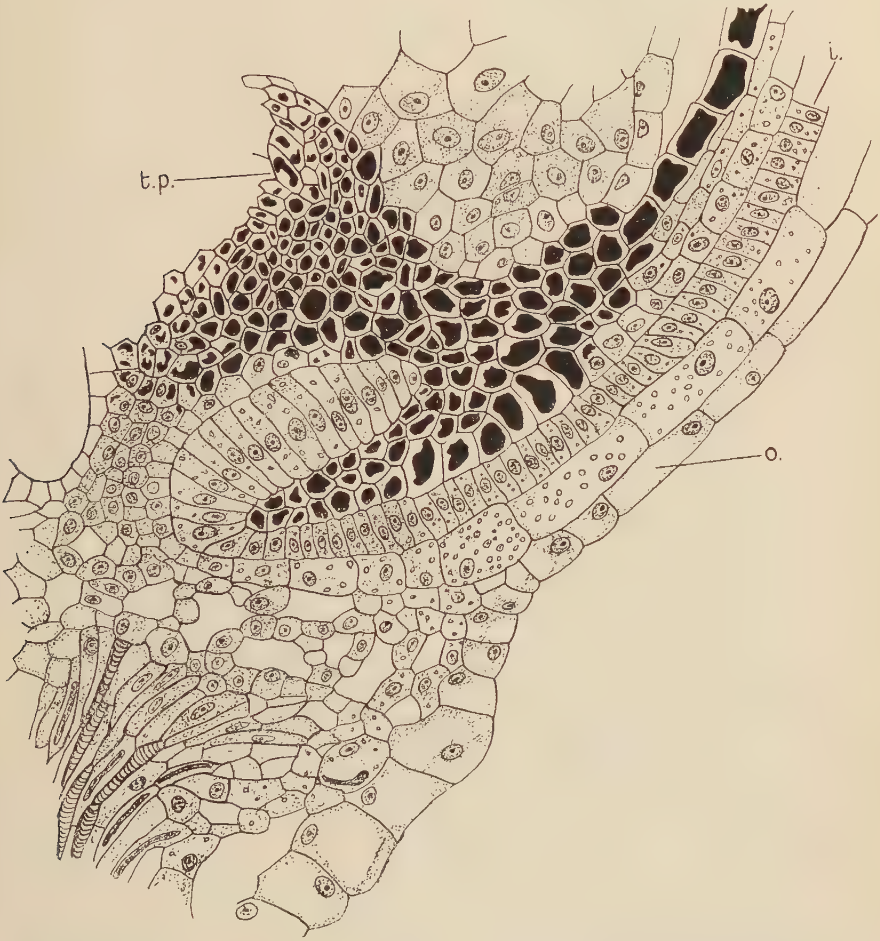
In the adult plant a balance is set up between the fungus and its host. It is debatable whether the minute embryo could set up this balanced relationship, and whether it could prevent the fungus from parasitizing it. The seed provides another example of mature cells being in contact with meristematic cells. When this occurred in the root, the meristematic cells were adversely affected by the presence of the fungus in the mature cells, and their metabolism was disturbed and cell-division ceased. However, the fungus never enters the seed proper; it only penetrates through the cells of the outer integument.

During the maturation of the ovule various modifications of the tissues occur. The column of cells at the chalazal end of the ovule (the column which is surrounded by the invaginated ring of columnar cells of the inner integument) is the first region to show signs of these changes. Each cell becomes filled with a tannin-like deposit. A dome-shaped area of nucellar cells immediately anterior to this column also becomes filled with tannin, as does the complete outer layer of nucellus cells. This means that the nucellus tissue, and in time the embryonic tissue too, is surrounded by an unbroken layer of tannin-filled cells. These changes mean that the base of the ovule is 'plugged' with an impenetrable tannin-filled barrier. The fungus which fills many cells of the funicle cannot pass into the ovule tissue or into the developing embryo (Text-figs. 4, 5).

The fungus, however, *can* pass from the funicle into the cells of the outer integument. Having entered these cells, penetration of the fungus into the tissues of the ovule is prevented firstly by the presence of the tightly packed thick-walled columnar cells of the inner integument, and secondly by the tannin layer inside this. The fungus could enter the embryonic tissues if it were present in the pollen grain; a great number of grains were examined for the presence of the fungus, but always with negative results.

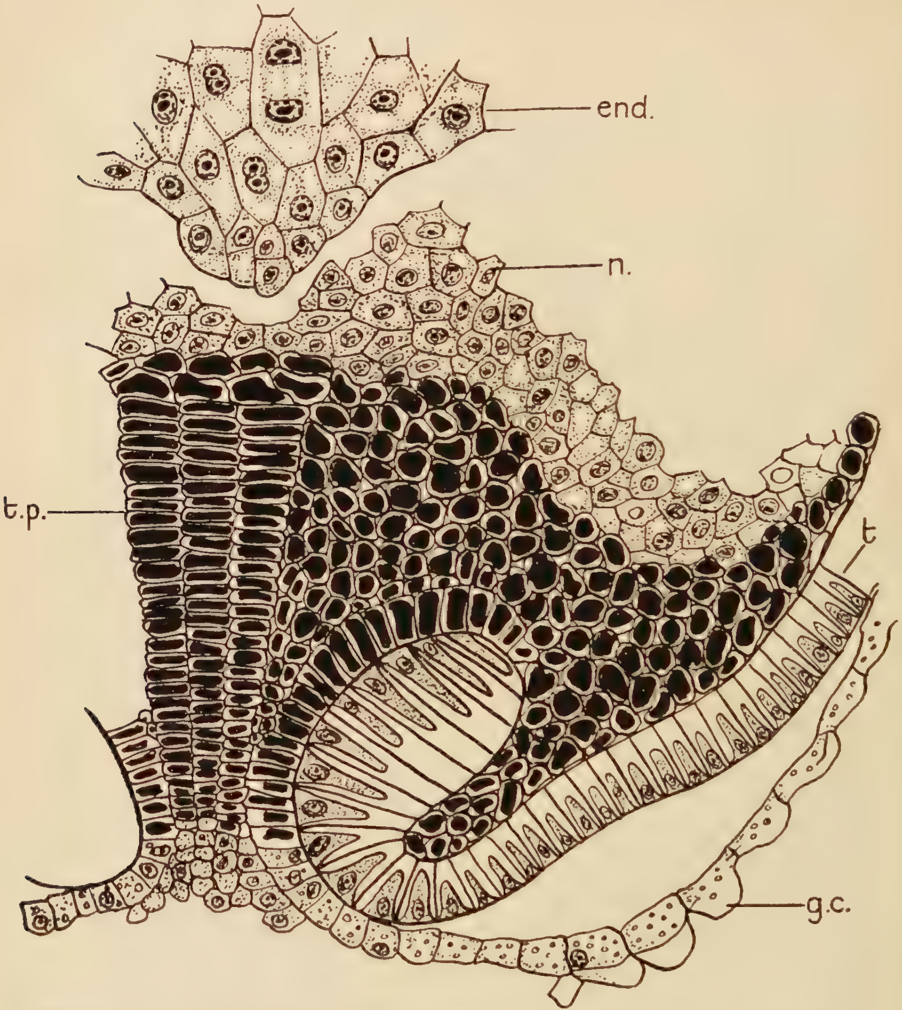
Maturation of the seed

As the seed matures, the outer cells of the outer integument become devoid of contents, and all that can be seen is the nucleus which clings very closely to the wall. The inner cells are still full of starch grains. During maturation of the seed much water is given off, and it is thought that at this stage the wall between the two layers of cells collapses, giving the single-layered coat made up of cells full of starch grains and containing the fungus resting on the thick-walled columnar cells of the inner integument (Text-fig. 6, B).



Text-fig. 4. L.S. Base of fully developed ovule of *Helianthemum chamaecistus*, showing the fungus in the vascular parenchyma of the funicle. Note the columnar cells of the outer layer of the inner integument. *i.* inner integument; *o.* outer integument; *t.p.* tannin plug.

In the mature seed the outer layer of the inner integument forms the important part of the seed-coat. These tightly packed columnar cells become thickened along the inner and along the four lateral walls, but the outer wall remains unthickened, and the characteristic U-shaped thickening results. This layer becomes deeply pigmented and forms the hard brown testa of the seed. The inner two layers of cells of this integument disintegrate completely. When the seed is dry, the outer coat, which contains the fungus, adheres very closely to the thick-walled columnar cells which constitute the seed-coat. When the seed is soaked in water this coat swells up and forms a thin, almost transparent film around the seed, with a small aperture at the micropylar and chalazal ends where the layer is obviously not complete. This is the layer to which the term *gelatinous coat* has been given.

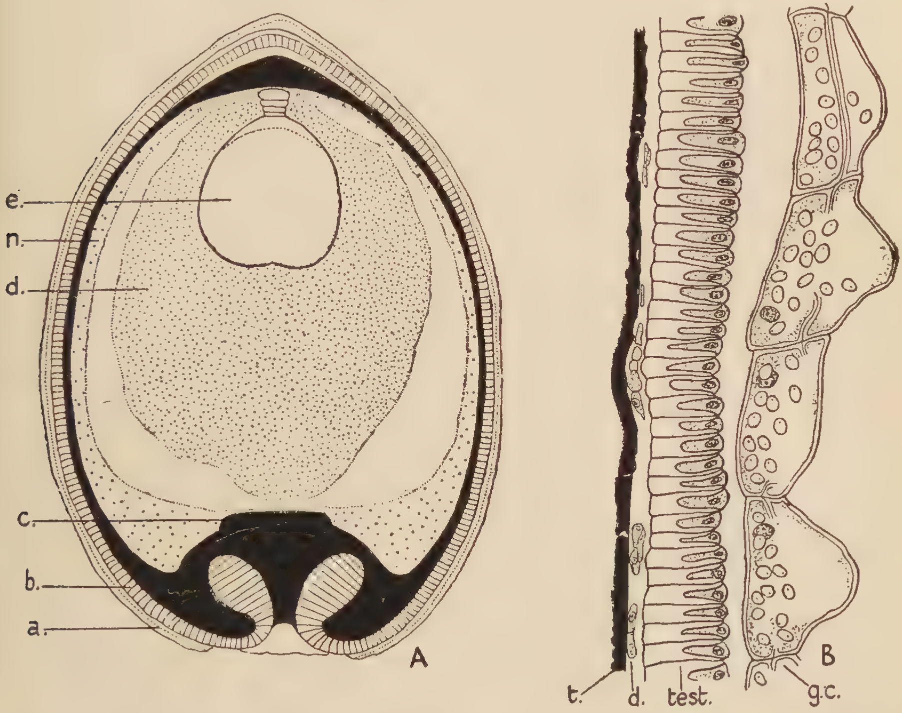


TEXT-FIG. 5. L.S. Base of seed of *Helianthemum chamaecistus*, showing the density of the tannin plug. *end.* endosperm; *g.c.* gelatinous coat; *n.* nucleus; *t.p.* tannin plug.

In a section taken longitudinally through the mature seed the following characteristic features can be seen (Text-fig. 6, A):

(*a*) An outer layer of unthickened unmodified cells, full of starch grains through which the hyphae ramify (the gelatinous coat).

(*b*) The testa, made up of thick-walled radially placed columnar cells with the characteristic invaginated ring at the chalazal end.



TEXT-FIG. 6. L.S. through the seed of *Helianthemum chamaecistus*. A. Plan of seed (for legend see text). B. L.S. through the testa and the gelatinous coat. *d.* disintegrated cells of the inner two layers of the inner integument; *g.c.* gelatinous coat; *n.* nucellus; *t.* tannin layer; *test.* testa.

(*c*) A complete layer of tannin-filled cells around the endosperm, one cell thick at the sides and apex but made up of many cells at the base, which seems to form an impenetrable barrier against the entry of the fungus.

(*d*) The endosperm tissue, with bi- and tri-nucleate cells.

(*e*) The embryo itself at the micropyle end of the seed.

The only part of the seed in which the fungus is found is the thin-walled gelatinous coat. It is clear also how the development of peculiar features, characteristic of this genus, prevents the penetration of the fungus into the tissues of the seed. *The embryo plant, with its surrounding endospermic tissue, is the only stage in the whole life-cycle which does not show a symbiotic relationship between plant and fungus* (cf. *Calluna* and *Casuarina*).

The fact that the fungus is found in the outer coat means that the embryo plant can, on germination, become infected as the root breaks through this coat.

This elaborate mechanism, which is apparently directed towards preventing the fungus from penetrating the embryo, ensures that fungal infection, and the consequent setting up of a symbiotic association, does not take place until the minute plant is capable of maintaining such an association.

GERMINATION AND GROWTH OF THE SEEDLING AND ITS REINFECTION BY THE FUNGUS FROM THE GELATINOUS COAT

It is desirable that all experiments with seedlings should be carried out under completely sterile conditions. One can then be certain that one is dealing with the fungus from the seed-coat, and not with saprophytic forms that may cling to the testa or may enter the root from the medium in which the seedling is growing.

Sterile seeds were obtained by dissecting open an almost ripe ovary under sterile conditions and removing the seeds into sterile containers. This method is the one described by Rayner (1915) in her work on *Calluna vulgaris*.

The greatest difficulty met with in this work was the fact that the percentage germination was often nil. McLennan (1935) points to a similar difficulty with seeds of *Epacris impressa*, and Rayner (1929) met with the same problem in experiments on the mycorrhizal relationship in the genus *Vaccinium*.

The seeds of *H. chamaecistus* are extremely hard, and to induce germination the method of puncturing each seed individually was adopted. This was done under sterile conditions, and care had to be taken to see that the gelatinous coat was not removed during the process. The punctured seeds were set on a variety of media, and the germinating seedlings were examined for the presence of the fungus. The gelatinous coats were also removed from the seeds at various stages in germination to observe the growth of the mycelium.

When the seeds are placed in, or on, the culture medium, the gelatinous coat swells up immediately. If this coat is removed a few days after germination, sculpturing of the starch grains is very marked, and this suggests that the starch is being digested. Whether this is one of the functions of the fungus is not certain: the fungal mycelium, however, has grown to a considerable extent. The root breaks through the ruptured portion of the testa within a few days of setting the seed. An interesting feature of this root is the absence of root-hairs. This lack of root-hairs has been recorded in other mycorrhizal plants; Rayner (1915, 1933) states that they are absent in *Calluna vulgaris* and in *Citrus*, and it raises the question whether this lack of root-hairs has any bearing on the mycorrhizal association. The root possesses an extremely long root-cap, the cells of which extend initially from the junction of hypocotyl and radicle to the tip of the root. In culture the cells of the piliferous layer and the outer layers of the root-cap are seen to come off in strips. These strips sometimes break off completely, but normally they remain attached to the plant, and give the appearance of multicellular root-hairs. The whole cortical tissue often becomes broken up by transverse breaks from piliferous layer to stele. It appears that the cells of the epidermis and cortex possess walls in which the calcium pectate layer is harder than is normally the case. This may be the result of the calcicolous habit. Normal growth of the cell is the result of the stretching of the wall when the cell becomes turgid. In this case, however, the walls are too deeply impregnated with calcium pectate, and are so hard that stretching does not occur, and as a result there is no increase in size of the cell. The failure of production of root-hairs can probably be attributed to the same cause. The elements of the stele in the same region elongate rapidly, however, and so we get a condition in which there is rapid growth inside the root, correlated with little or no growth of the outer tissues. This must result in the breaking of the outer cells; sometimes the epidermis only is affected, but at times many layers of cortical tissue are broken up too. The possible significance of this phenomenon will be discussed later.

When the seedling root is examined for the presence of the endophyte, small strands of hyphae can be seen in the cells of the cortex. Infection must have taken place from the seed. This infection of the young root by the mycelium in the gelatinous coat occurs soon after the radicle has emerged. Before the hyphae grow out and penetrate the root, they grow and increase in length at the expense of the starch grains in the cells. When the mycelium grows out of the coat, the root is already a few millimetres long; this means that the region of the root which comes in direct contact with the hyphae is that in which tissue differentiation has commenced. The pro-meristem cells do not appear to come in contact with the mycelium, and it is the region immediately behind the pro-meristem which becomes infected by the fungus from the coat. It is extremely difficult to follow the hyphae in the seedling; first examination almost invariably brings negative results. The hyphae in the gelatinous coat are septate, but when they enter the root septa become rare or absent. They penetrate the cell-walls, and where this happens there is a slight constriction. The fungus is mainly intracellular, and it penetrates from cell to cell, entering new cells at the corners. From the cortex it passes into the elongated thin-walled pro-cambial elements. As the first phloem and xylem cells are differentiated from the pro-cambial cells, the hyphae gain access to these tissues. The protoxylem and protophloem cells are slender and long, and possess thin cellulose walls: these cells are used by the fungus as a means of rapid spread through the plant. As the pro-meristem of the root divides and as new tissues are formed, the fungus spreads into these regions as they are differentiated. Thus, from the original point of penetration, the fungus now extends into all new root tissues as they are formed. Initial infection took place at a point just below the junction of root and hypocotyl. From here the fungus spreads into the hypocotyl and eventually into the plumule. In the shoot it extends as far as the region of tissue differentiation just behind the promeristem at the apex. As in the root, it keeps pace with the growth of the apical meristem and enters new tissues as they arise. The hyphae usually pass into the leaf with the phloem parenchyma, and they are thus found in the dorsal areas of the leaf.

In this way the fungus spreads throughout the seedling and thereby gains access to all tissues of the plant as they are formed. In the older tissues of the root the fungus will, at a later stage, undergo a process of digestion, and the hyphae in the mature vascular elements begin to degenerate. In this way contact between root and shoot mycelium is broken and in the adult plant it is difficult to find any trace of the endophyte in the older tissues. The fungal mycelium remains active in the roots of the seedling throughout the first winter, and it is from the cortical cells in these regions that the young laterals are infected as they arise in the following season. On the whole, digestion of fungal hyphae on a large scale does not occur until the end of the second season of growth.

GENERAL DESCRIPTION OF THE FUNGUS

It is extremely difficult to give a morphological account of the fungus, as its form varies considerably and seems to depend on the type of tissue in which it is found, and on the period of the year. Rayner (1915) stated that the mycelium in the tissues of *Calluna vulgaris* was distinguished from that outside by the smaller diameter of the hyphae. She also pointed to the variation in diameter within the tissues. McLennan (1926) showed that in *Lolium* the walls of the hyphae, which were thick and brown outside the root, were thin and colourless inside, but that the dimensions increased greatly. This variation is seen in *H. chamaecistus* also. In the gelatinous coat of the seed we find a branching septate mycelium, with fairly dense cytoplasm and numerous vacuoles. The hyphae are about 4μ in diameter. These are the hyphae which penetrate the radicle on its emergence. In the root septa are rare, but the hyphae still contain dense cytoplasm and there is very little change in their diameter. In the regions where there is active growth of the mycelium, the hyphae maintain these characteristics. It is in the mature tissues that changes in hyphal form are seen. Partial digestion of the intracellular mycelium in the root results in a considerable increase in the diameter of the hyphae, and the cytoplasm becomes much less dense. The hyphae are gradually emptied of all their contents. The intercellular mycelium does not show this great increase in diameter, although the hyphae now measure 8μ across. The ectotrophic hyphae formed at this period have thick pigmented walls, which do not take up the stains. They have numerous cross-walls and they measure from 5 to 8μ across.

There is no indication of the presence of vesicles, arbuscules, and sporangioles. The fungus has only been seen in the hyphal form: there are no reproductive bodies. It has not yet been possible to grow the fungus in pure culture, notwithstanding the use of a great variety of media.

FUNCTION OF THE FUNGUS IN THE PLANT

Since the classical researches of Frank (1885) much discussion has centred around the relationship which exists between the two components of a mycorrhizal association, particularly in the case of endophytes. Bernard (1904) called the endophyte in the orchids a parasite, and suggested that it attacked the embryo plant at an early stage and persisted in the adult as a chronic, but relatively benign condition of disease. Burges (1936) states that the association between the fungus and the roots of higher plants should be regarded as one of controlled parasitism, as it has no 'mutualistic significance'. He says the fungi are weak pathogens whose activity is curbed by the reaction of the host cells. Later (1939) he states that 'breakdown of the hyphae in the root is probably the most striking phenomenon associated with mycorrhizal infection, and is responsible to a large extent for the separation of mycorrhizal infection from the more usual forms of parasitic invasion'. He goes on to say that breakdown results in the liberation of fungal proteins into the cell and thus may represent a gain to the plant. Against this theory of the controlled parasitic nature of the endophyte, we find a mass of evidence pointing to the symbiotic nature of the fungus. Many workers have suggested the function of *nitrogen fixation* for the endophyte. Ternetz (1907) found that fungi isolated from the roots of Ericaceous plants were able to fix atmospheric nitrogen. Rayner (1915), experimenting with pure culture seedlings of *Calluna vulgaris* inoculated with the endophyte and grown under sterile conditions in media lacking combined nitrogen, found that they were green and healthy, and grew at the same rate as the controls. Neilson-Jones and Smith (1928) cultivated the fungus from *Calluna* and concluded that it could utilize the molecular nitrogen of the air.

Another theory of the function of the fungus suggests that some sort of *exchange of food material* takes place between the endophyte and its host. Many workers suggest that the fungus supplies an enzyme which renders soluble the insoluble materials stored in the cells, while it has also been shown that the host plant may produce an enzyme to bring about the digestion and subsequent utilization of fungal material in the cell. Knudson (1922) suggested that the activating effect of the fungal mycelium in the orchids was due to the production of enzymes which acted upon the insoluble carbohydrates present in the seed tissue. Endrigkeit (1937) also supports this theory and shows that the disappearance of starch from the root cells of the host is a reaction to fungal infection. McLennan (1926) showed that in *Lolium* the fungus itself was digested by the root cells and was used 'as a source of carbonaceous nutriment'. Fat or oil which accumulates in the hyphae is, at a certain stage in the association, given up to the host cell through the disintegration of the sporangiole wall. That this is utilized by the higher plant is evident by its removal from the cells. McDougal and Dufrenoy (1946) suggest, as a result of cytochemical tests on the cortical cells, that carbohydrates, phospholipids, and proteins are metabolized in the hyphae of the fungus, and are then transported to the cortex of the higher plant.

Muller and Schopfer (1937) and Robbins (1939) suggest that symbiosis may be intimately related to the ability of one member of the association to *supply growth factors* to the other. Burgeff (1934) was the first person to work on this line; he suggested that vitamin autotrophic micro-organisms were capable of producing accessory growth factors which were supplied to the host. He found (1936) that the dead symbiont of the orchids was quite as effective in stimulating growth as the living fungus, and he showed that the active principle, normally formed by the fungus, was an accessory growth factor resembling Bios II. The orchids of the *Vanda* group are heterotrophic for this substance at least in the seedling stage. This inability of the plant to synthesize the growth factor is made good by the autotrophic fungus. Schaffstein (1938) showed that the seed of *Phalaenopsis* contained very little vitamin, although the green parts of the mature plant were relatively rich in vitamin. It is thus only during the early stages that the plant requires the growth substance which the fungus can synthesize. Noggle and Wynd (1943) obtained good germination and excellent development of seedlings of *Cattleya* when nicotinic acid was added to the medium, and suggested that this was the factor that the seedling was unable to synthesize, and which under normal conditions was supplied by the symbiotic fungus.

In view of previous work carried out on the function of the fungus in many kinds of symbiotic associations, three main lines of investigation were followed to ascertain the probable function of the endophyte of *H. chamaecistus*. These were the possible fixation of atmospheric nitrogen, exchange of food substances, and the supply of accessory growth factors.

If the fungus is isolated one can study its metabolism under controlled conditions. If this is impossible one has to fall back on microscopic examination and upon a study of the host's metabolism in relation to its symbiont. Attempts must also be made to grow the plant from sterile seeds by removing the gelatinous coat and thus obtain a fungus-free plant. To the medium in which this plant is growing various substances must be added to ascertain whether one or a combination of these substances is able to replace the fungus. In this way it may be possible to deduce the probable function of the fungus in the plant.

Experiments on the possible nitrogen fixation by the fungus

Seedlings grown without the fungus germinate successfully, but growth ceases at a certain point. The plumule does not emerge and no branch roots are formed. Seeds from which the fungus has not been removed develop normally. It appears that the presence of the fungus is essential for successful growth. To ascertain whether the need for the fungus was bound up with the power to fix atmospheric nitrogen, seeds with and without the fungus (i.e. those with and without the gelatinous coat) were grown in a full nutrient solution with an inorganic nitrogen supply, with an organic nitrogen supply in the form of asparagine or glycine, and lastly in a solution from which every source of nitrogen had been removed. An attempt was made to provide a basic solution which included all essential substances for plant growth. In this way it was felt that lack of growth could not be put down to any cause other than the lack of nitrogen supply. The solution used was that recommended by White (1943) for tissue-culture work.

In all cases seeds germinated successfully and reached the stage of production of hypocotyl and cotyledons, and an unbranched primary root. From this point onwards growth differed greatly. The seedlings grown *with* the fungus, on a medium lacking combined nitrogen in any form, failed to continue their development and after some time the root became brown and growth ceased. Germination occurred because the plant was able to rely on the nitrogenous supply in the seed; when this was utilized, the seedling was dependent on an *outside* supply of combined nitrogen; without this no growth occurred despite the presence of the fungus. Seedlings grown without the fungus but with an inorganic or organic nitrogen supply also failed to develop beyond a certain point. Thus a supply of combined nitrogen cannot replace the fungus, and the latter is incapable of fixing atmospheric nitrogen and supplying it to its host.

Experiments on the possible exchange of food between fungus and host

Most of this work was carried out with roots because of the greater intensity of hyphae in these regions. Since etching of the starch grains in the cells of the gelatinous coat had been observed, it was thought that the fungus might play some part in making insoluble carbohydrates available to the plant. Observations were therefore made on the distribution and occurrence of carbohydrates in the cells.

Seedling roots were examined, and it was found that although starch was absent in normal symbiotic roots, in those plants which were grown without the fungus there was quite a heavy deposition of grains in the cells. Older roots were also examined, and where there was dense endotrophic infection there was no starch. Burgeff (1909) showed that in orchids, too, the appearance of an intracellular mycelium coincided with the disappearance of starch from the invaded cells.

The presence of the fungus appears to be correlated with the absence of insoluble carbohydrates. Is the function of the fungus that of supplying the plant with soluble carbohydrates? Does this mean that the fungus can be replaced, as Knudson suggests for the orchids, by supplying the plant with sugars? Experiments on symbiotic growth showed that provision of sugar alone was not enough to bring about successful development, although growth did show some improvement. We can say with some certainty that the fungus benefits the plant as a result of its action on insoluble carbohydrates: this, however, is not its only function. It is probable that the fungus utilizes some of the soluble carbohydrates for its own metabolism.

Tests for the presence of proteins and fats in the cells and hyphae were carried out. There appears to be no relationship between the presence of proteins in the cells and the presence of the endophyte. There is no storage of fats in the cells of the plant.

At the end of the summer shrivelled empty hyphae are seen in some of the root cells which are also devoid of contents. It is likely that soluble substances diffuse through the thin fungal membrane into the cell and are transported directly to the growing parts of the plant. It is obvious that at this period the endophyte is used in some way by the root as a source of nutriment.

Experiments to ascertain whether the fungus relieves a possible avitaminosis in the plant by supplying one or more essential growth substances

In experiments on asymbiotic growth of seedlings, thiamin, biotin, pyridoxine, and nicotinic acid were added alone and in various combinations to the basic medium. It was found that the addition of thiamin (1 in 20,000,000) resulted in successful germination and development of seeds devoid of the fungus, the plumule of the seedling emerging and the primary root branching extensively. Kögl and Haagen-Smit (1936), working on detached pea embryos, showed that thiamin was essential for development and that it markedly affected the growth of the roots. It is root growth which suffers in asymbiotically germinated seeds of *H. chamaecistus*. These results suggest that without the fungus the plant suffers from a lack of thiamin, but that in nature this is made good by the presence of a fungus which is auxo-autotrophic for this substance. It leads us to the opinion that one of the functions of the fungus is to relieve this avitaminosis in the seedling by supplying it with thiamin. Experiments to ascertain whether thiamin was present in the seed and in the mature tissues were carried out and will be described, together with full details of asymbiotic growth, in a later paper. It can be deduced with some certainty from this work that one of the functions of the fungus in *H. chamaecistus* is to provide the plant with thiamin.

Experiments on the stabilizing of the calcium metabolism of the host by the fungus

Under cultural conditions the primary root of seedlings and the branch roots of older plants show a marked stripping of the epidermis, and numerous transverse breaks in the cortex. Phenomena such as these have been recorded in many plants suffering from an excess of calcium in the culture solution (Cormack, 1935), and it is suggested that this is due to the fact that the walls become unduly impregnated with calcium pectate. *H. chamaecistus* is a calcicolous plant and an excess of calcium in the cells is to be expected. There is a heavy deposition of calcium oxalate crystals in the cells, these crystals often being found in association with fungal hyphae. We know that fungi frequently produce oxalic acid as a by-product of their metabolism. Does this fungus give off oxalic acid, which combining with the calcium taken in by the plant, removes the effect of an excess of calcium and stabilizes the calcium metabolism? It is difficult to say whether the oxalic acid in *Helianthemum* is produced by the fungus. It may be formed in other ways. Calcium oxalate crystals, however, are only found in those seedlings grown with the fungus, and it is reasonable to suppose that there is some connexion between fungal infection and the presence of crystals.

Seeds with and without the fungus were germinated in an inorganic medium with calcium and one without, and the seedlings were examined for the presence of excess calcium in the cell sap, calcium oxalate crystals in the cells, stripping of the root cap and piliferous layer, and for transverse breaks in the cortex. Seeds were tested with picrolonic acid for the presence of calcium, and cultures were set up with isolated embryos.

TABLE I

Effect of Calcium on Symbiotic and Asymbiotic Seedlings

	Conditions of growth					
	With fungus		Without fungus		Embryo only	
	Nutrient plus Ca	Nutrient minus Ca	Nutrient plus Ca	Nutrient minus Ca	Nutrient plus Ca	Nutrient minus Ca
Excess Ca in cell sap	−	−	+	±	+	−
Calcium oxalate crystals	+	±	−	−	−	−
Stripping	±	−	+	±	+	±
Breaks in cortex	−	−	+	−	+	−

+ Positive or present. — Negative or absent. ± Trace.

The results, shown in Table I, demonstrate that in the absence of the fungus there is an excess of calcium in the cell sap, and that stripping of the cells and breaks in the cortex occur as a result, presumably, of the undue impregnation by calcium of the pectic wall of the cell. The slight deposition of crystals in those symbiotic plants grown in a solution without calcium is the outcome of the presence of calcium in the seed. When the fungus is present there is slight stripping of the piliferous layer during the early stages of germination, but once the fungus begins to ramify through the cells of the root this phenomenon ceases: there are no breaks in the cortex.

The fungus appears to play some part in stabilizing the calcium metabolism of the plant, owing to the deposition of calcium oxalate crystals and the consequent removal of excess calcium from the cell sap, thus preventing its combination in too great a quantity with the pectic substances in the wall.

DISCUSSION

The term 'cyclical symbiosis' first used by De Jongh lies at the core of this work on *Helianthemum chamaecistus*. All aspects of the problem are bound together in these two words. Is this fungus a cyclical one? Does it really pass from one generation to another through the seed, and is its presence of any value to the plant at one or at all stages in its life history?

When a fungus permeates through the tissues its presence is frequently deleterious to its host; if it is present in the seed it often parasitizes the young seedling at germination. Examination of the mature tissues of *H. chamaecistus* showed that the presence of the fungus was not harmful to the plant: it was more difficult to prove that it was beneficial. Germination and subsequent development of the seedling, however, is dependent on the presence of the fungus in the seed. Without it the plant ceases to grow beyond a certain stage, the plumule does not emerge, and the primary root does not branch. It seems therefore that during germination the presence of the fungus is essential. In orchids infection of the seedling takes place from the soil; here in *Helianthemum* the fungus is present in the seed and infection of the young seedling is thus assured. The fungus enters the seedling root and penetrates from here into the hypocotyl and plumule; it keeps pace with growth and differentiation at the apices and enters new tissues as they arise. The identity of the root fungus with that in the shoot is therefore seen very clearly in the seedling, though in the mature plant the connexion between the two is usually severed. The development of the fungus in the shoot was followed: it was seen in an active state in the young vascular parenchyma elements just behind the apical meristem and as new tissues were formed the fungal hyphae were seen to penetrate them. In this way the fungus gains access to the floral meristem and later the ovary wall. Its presence in the funicle of the seed was recorded in every specimen examined, and in no case was the gelatinous coat of the seed found to be devoid of a branching, septate mycelium. These facts are considered to be proof that the fungus of *Helianthemum chamaecistus* is a cyclical fungus, and that the host plant is a dual organism, harbouring a fungus in the seeds so that the association becomes hereditary.

The presence of the fungus has been shown to be essential during early stages of growth. Is it essential in the mature plant too, or is it only tolerated here because its presence in the seed is essential and because this is the only way for its presence in the seed to be assured? Examination of the root throughout the year suggests that its presence is beneficial to the plant. In spring and summer it ramifies through the cells, and enters new roots as or soon after they arise. In autumn, however, the hyphae are seen as swollen empty structures in the cells. It is reasonable to suppose that the plant has utilized the fungal material in the cells by means of digestive enzymes. In the stem there are no signs of digestive processes; here the fungus seems to be tolerated so that it may gain access to the seed.

The higher plant thus benefits in two ways at least from the presence of the fungus. Successful germination only occurs when it is present, and the endophyte in the root is digested and used as a source of nutriment. The fungus must obtain soluble food from the plant, for it has no contact with the soil except for one short period when it passes through an ectotrophic phase. It also solves, as a result of this association, the problem of the perpetuation of the species. It no longer needs to form reproductive organs; it reproduces its own species by the simple means of entering every seed in the ovary. Successful germination of the seed results in the renewal of growth of the fungus which remains dormant in the seed. When germination fails, then the fungus dies. It never lives outside the plant and it is doubtful whether it is capable of doing so. The metabolic and reproductive cycles of the host and the endophyte are both intimately bound one with the other, and we appear to have an association which is symbiotic in the true sense of the word.

SUMMARY

1. The presence of a branching septate fungal mycelium has been recorded in the outer coat of the seeds of members of the family Cistaceae, and its life-cycle elucidated in *Helianthemum chamaecistus*.

2. The fungus enters the seedling from the seed and spreads throughout the plant, keeping pace with the differentiation of tissues at the apices, and entering new tissues as they arise.

3. It enters the ovary, and passes up the funicle into the gelatinous coat (the outer integument) of the seed. It is prevented from entering the seed proper by a plug of tannin-filled cells at the base of the ovule and by an unbroken layer of similar cells inside the testa.

4. The embryo and endosperm are the only parts of the plant which do not contain the fungus.

5. The fungus passes from generation to generation through the seed. The relationship between fungus and plant is a hereditary one.

6. Two types of roots occur: normal long roots and *tuberous roots*. These latter are infected by the fungus while inside the parent cortex. This results in the swelling of the cells and in the loss of the meristematic activity.

7. In autumn the fungus in the older roots undergoes digestion, the hyphae swell, break up into small pieces, and lose their contents. It is not digested in the new long roots, and thus serves as a source of infection in the following spring. The intercellular mycelium of the tuberous root grows out to the surface in the autumn, forming a mat of ectotrophic growth.

8. Growth of the seedling ceases at a certain point in the absence of the fungus: the plumule does not emerge and no branch roots are formed.

9. It is suggested that one of the functions of the fungus is to provide thiamin to the seedling, thus relieving an avitaminosis of the plant. The fungus does not fix atmospheric nitrogen.

10. The term *cyclical symbiosis* has been used to describe the association between the seed-borne fungus and the higher plant.

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In a further paper it is hoped to give an account of experiments in which successful asymbiotic germination was brought about, and details of attempts to isolate the fungus.

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EXPLANATION OF PLATE

Illustrating J. G. Boursnell's article 'The Symbiotic Seed-borne Fungus in the Cistaceae'.

Fig. 1. Fungal mycelium in the cells of the gelatinous coat of the seed of *Helianthemum chamaecistus*. (× 100.)

Fig. 2. Hyphal coil in cortical cell of the root of *Helianthemum chamaecistus*. (×650.) *h*. normal hypha; *h.c.* hyphal coil.

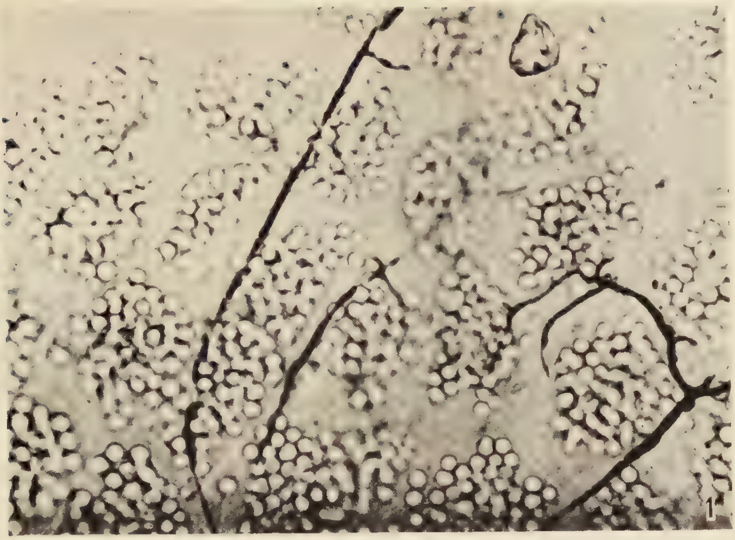

FIG. 1. Fungal mycelium in the cells of the gelatinous coat of the seed × 100.

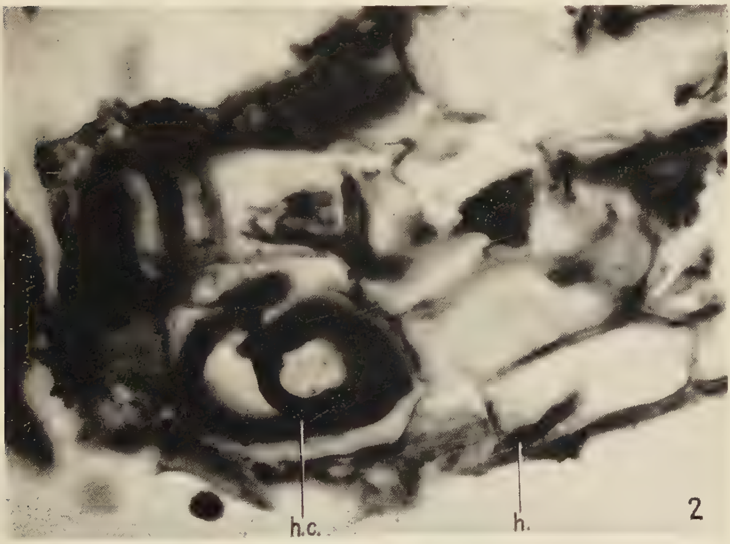

FIG. 2. Hyphal coil in cortical cell of root × 650.

J. G. BOURSNELL

Symbiosis of Leguminous Plants and Nodule Bacteria

III. Observations on the Growth of Soya Bean in Water Culture

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INTRODUCTION

ALTHOUGH the technique of water culture has been employed extensively with normal autotrophic plants, it has been utilized relatively infrequently with nodule-bearing leguminous plants, and when, in connexion with experiments already described (Bond, 1941), it became necessary to grow nodulated soya-bean plants in water culture, it was found that relatively little guidance as to procedure could be obtained in the literature. As the result of experience then gained, some aspects have been investigated further, and in the present paper experiments extending over several years are described in which particular attention has been paid to the effects of aeration and the oxygen factor in nodulated soya-bean plants growing in water culture. Data are also presented on growth in different culture solutions and on the pH relation. The inclusion in some of the experiments of plants free of nodules and supplied with combined nitrogen, in addition to nodulated plants in nitrogen-free solution, assists in the determination of how far any effects observed are peculiar to plants associated with, and dependent on, nitrogen-fixing bacteria.

A review of the literature on the effect of forced aeration of water cultures of non-leguminous plants is included in the paper by Erickson (1946). In many instances the aeration of water cultures of ordinary type (i.e. with the cover not air-tight and with an air-space above the solution) has been found to benefit plant growth; in other cases no benefit has followed. Such discrepancies doubtless reflect the varying oxygen requirements of different species and may also have arisen from variations in experimental detail, such as the shape of the culture vessel. Several previous investigations on the effect of aeration in which soya-bean plants (non-nodulated and supplied with combined nitrogen) have been used are of special interest and will be considered in the Discussion.

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The effect of forced aeration of water cultures of nodulated leguminous plants was investigated in the experiments of Virtanen and von Hausen (1936) in which peas were grown aseptically in 3-litre Woulff's bottles containing nitrogen-free Hiltner's solution. In non-aerated cultures abundant nodules developed but remained relatively small; the average fixation of nitrogen was 10 mg. per plant, with a dry weight of 1·03 g. With aeration nodules were larger, fixation was 73 mg. per plant, and dry weight 2·64 g. The plants in a single culture of uninoculated peas supplied with excess of ammonium sulphate, without aeration, were considered to show good growth, gave a dry weight of 1·16 g. and a nitrogen content of 80 mg. per plant. The results of the experiment were ascribed to the effect of the oxygen content of the medium, which was considered to be adequate without aeration for root but not for nodule development and function, the latter having, it was concluded, a relatively high oxygen requirement.

Evidence on the last question is also provided by the investigations of Wilson and Fred (1937), in which clover plants were grown in sand culture totally enclosed in large bottles, the atmosphere within these being adjusted to contain different partial pressures of oxygen. At the lowest partial pressure the proportion of oxygen present was only $\frac{1}{16}$th of that in normal air. The response of nodulated plants in nitrogen-free medium was similar to that of non-nodulated plants receiving ammonium nitrate. Hence there was no evidence of a special requirement of oxygen by any part of the nodulated plant, a finding which is not readily reconciled with that of Virtanen and von Hausen (above).

Direct observations are available on the oxygen uptake of nodules and of their respiratory activity as compared with roots, but these observations also are discrepant. Allison (F. E.) et al. (1940) presented extensive observations by manometric methods on the oxygen uptake and carbon dioxide production of excised nodules and root fragments supplied with glucose. The rate of respiration of nodules was not appreciably higher than that of root tissues. Demolon and Dunez (1943) in observations on pulped nodule and root tissues found that the rates of carbon dioxide production were similar. Asprey and Bond (1941), on the other hand, in manometric observations on excised nodules and roots of soya-bean grown in water culture, found the oxygen intake per unit dry weight of nodules to be 2–3 times that of roots, with comparable differences in respect of carbon dioxide evolution.[1] Bond (1941), in observations on intact plants of soya bean in water culture, concluded that the evolution of respiratory carbon dioxide per unit dry weight of nodule tissues was approximately 3 times that from root tissues. It may be pointed out that the observations of high intake of oxygen in the presence of excess of that gas does not necessarily mean that nodules will be unable to function efficiently under reduced oxygen tension. Anaerobic mechanisms may be present which come into play under such a condition.

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[1] For a description of the respirometer used in these observations see Asprey, G. F., 1948: An Apparatus for following the Course of Gaseous Exchange by Living Tissue. Proc. Roy. Soc. Edin., B. lxiii, 163–76.

METHODS

The Manchu variety of soya bean has been used in these experiments, seed being kindly supplied by Dr. W. J. Morse, United States Bureau of Plant Industry. Seeds of closely similar weight, after surface-sterilization, were sown in troughs of sand. At the stage when the plumule was unfolding, usually after about 12 days' growth, uniform seedlings were transplanted to static, non-flowing water culture in wide-mouthed glass jars of capacity 2½ litres. The jars were 20 cm. in height, 9 cm. and 15 cm. in diameter at the mouth and below the shoulder respectively, and were equipped with loose-fitting waxed teak tops.

The culture solutions used in the experiments have been based on a nitrogen-free form of Crone's solution. Bryan (1922) reported that the Crone medium (soluble portion only) was satisfactory for the cultivation of nodulated soya bean. Various formulae for the Crone medium may be found in the literature. The original formula devised by von der Crone (1902), modified here to a nitrogen-free form, is shown in the accompanying table, together with other formulae relevant to the present experiments. Quantities are g. per litre.

	Original.	Used by Bryan.	Formula A.	Formula B.
KCl	0·75*	0·75	0·75	0·75
$CaSO_4 \cdot 2H_2O$ . . .	0·5	0·19	0·19	0·25
$MgSO_4 \cdot 7H_2O$ . . .	0·5	0·19	0·19	0·25
$Ca_3(PO_4)_2$ . . .	0·25	0·19	0·19	0·25
$Fe_3(PO_4)_2 \cdot 8H_2O$ . .	0·25	0·19†	0·19	0·25

* Replacing 1 g. KNO_3 of Crone's formula.
† $FePO_4$ was used instead of the ferrous salt.

Of the experiments described in the present paper, in those of 1946 formula A was used, while in 1947–8 formula B was substituted. It was believed at the time that formula B (Miller, 1938, p. 242) was the true Crone formula, but subsequent reference to the original paper of Crone (loc. cit.) showed that this was not the case. Tap-water (very low in available nitrogen) was employed in the preparation of the solutions.

Prior to use the solution was autoclaved at 20-lb. pressure for 15 minutes, and then stored in glass tanks until required. The pH of the autoclaved solution was 6·2. In some cases only the clear supernatant solution, obtained after the autoclaved mixture had stood in the storage tanks for 24 hours or more, was used, separation being effected by siphoning. In other experiments the medium was thoroughly stirred before use so that an appropriate amount of sediment was included, giving what will be termed the 'complete' solution. In a few cases what will be termed an 'intermediate' solution has been employed, and was prepared by adding 125 c.c. of 'complete' solution to each culture jar and then making up to 2½ litre with 'supernatant' solution. Minor elements (boron, manganese, zinc, and copper) were supplied to all jars in the form and amounts recommended by Loomis and Shull (1937, p. 60).

At weekly or fortnightly intervals (as indicated later) the contents of the culture jars were replaced with fresh solution. Sterile tap-water was added daily to maintain the solution 1–2 cm. below the teak tops.

Immediately after the young plants had been transferred to water culture, certain jars were inoculated with an effective strain of the soya-bean nodule organism (Wisconsin strain No. 9 in the first experiment of 1940, No. 505 in subsequent experiments). The remaining jars (apart from controls) were supplied with combined nitrogen in the form of sodium nitrate, which was added at frequent intervals in amounts estimated to be in excess of the requirements of the plants.

For aeration use has been made of two sources of air. In some cases an electric air-pump, compressing greenhouse air, was employed, the air being supplied for 16 hours out of each 24 hours, during hours of daylight. Alternatively air from commercial cylinders[1] was used, and in this case was supplied continuously to the plants. Dispersal of the air stream was obtained by fitting to the lead-in tube in each jar a cubical aerator of porous porcelain (2·5 cm. edge) of a type used for aquaria. The rate of flow was in general of the order of 1 litre per hour, but was varied somewhat according to the stage of development of the plants and the results of oxygen determinations (below), the object being to maintain oxygen tensions in the culture solution as near as possible to equilibrium values for water freely exposed to air.

Measurements of pH were usually made by glass electrode on samples withdrawn from midway down the jars. In certain experiments steps were taken to stabilize pH, which, as shown later, otherwise falls below the original value of 6·2 in jars of nodulated plants in Crone's solution. The procedure was to add daily to each jar sufficient N/10 NaOH to bring the pH to approximately 6·5.

Determinations of dissolved oxygen in the culture solution were made by the Winkler method, using the 10-c.c. syringe-pipette described by Whitney (1938), with precautions to avoid the introduction of manganous chloride into the culture jars. By fitting a cannula of suitable length, samples were withdrawn from levels midway down or near the base of the jars (9 cm. and 15 cm. respectively from the surface of the solution). All samples were taken during hours of daylight, and not less than 2 days after the weekly or fortnightly renewal of the solution. In order to facilitate comparison of oxygen data, results have been expressed as a percentage of the oxygen content of water in equilibrium with the atmosphere at the temperature and pressure prevailing at the time of the determinations, the data of Winkler (1889) being utilized for these calculations.

In the experiments, which were carried out in unheated greenhouses, cultures receiving the various treatments were, so far as was practicable, arranged in random manner on the benches, and positions were changed at frequent intervals. The experiments were continued for 13–16 weeks, by which time the plants were at the stage of forming young pods. Harvesting was then carried out, dry weights being obtained by heating the plant material at 95° C. and total nitrogen by Kjeldahl method.

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[1] It is understood from the British Oxygen Co. Ltd. that this air is pumped in a water-lubricated compressor.

EXPERIMENTAL RESULTS

1940 experiments. In two small-scale experiments the 'supernatant' Crone solution was employed, though separation from the sediment was probably less complete than in later experiments. The solution was renewed fortnightly and there was no adjustment of pH. The air-stream supplied to certain jars was provided by electric pump.

Results are presented in Tables I and II, and show that aeration increased nodulation, dry weight, and nitrogen fixation[1] in nodulated plants, while growth of combined nitrogen plants (Table I) was equally good with or without aeration. Determinations on samples of culture solution drawn from midway down the jars of the second experiment when the plants were 15 weeks old showed oxygen contents in aerated jars to be 90–95 per cent. and in non-aerated jars 60 per cent. of equilibrium figures.

TABLE I

Harvest Data for 1940 Experiment, Growth Period 15 May–9 August (data are per jar of 3 plants)

	Jar No.	No. of nodules.	Dry wt. of nodules (g.).	Dry wt. of roots (g.).	Dry wt. of tops (g.).	Total dry wt. of plants. (g.).	Total N content of plants (mg.).
Nodulated Plants							
Non-aerated	1	221	1·25	0·90	5·08	7·23	155
	2	236	1·30	1·01	5·64	7·95	158
	3	122	0·71	0·70	3·76	5·17	93
Aerated	4	408	1·75	1·02	9·80	12·57	395
	5	491	1·81	0·98	9·75	12·54	418
*Non-nodulated Plants**							
Non-aerated	1	—	—	5·25	22·74	27·99	403
	2	—	—	5·83	21·55	27·38	428
Aerated	3	—	—	5·64	24·58	30·22	432
	4	—	—	4·74	25·53	30·27	433
	5	—	—	4·17	19·55	23·72	418

* Supplied with a total of 500 mg. nitrate-nitrogen per jar.

1947 experiment. Both 'supernatant' and 'complete' Crone solutions were used with weekly renewal, with two sources of air. Daily adjustment of pH to 6·5 was applied to all jars of nodulated plants. Data obtained at harvest are given in Table III. With nodulated plants the 'complete' solution gave

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[1] Fixation of nitrogen per jar of 3 nodulated plants in the experiments described in this paper is obtained by deducting 20 mg. from the nitrogen contents shown in the tables. This deduction is based on the examination of control jars of non-nodulated plants not supplied with combined nitrogen. There are good reasons for assuming that there was no loss of fixed nitrogen by excretion in the present experiments (Bond, 1941, p. 321).

TABLE II

Harvest Data for 1940 Experiment, Growth Period 12 June–24 October, all Nodulated Plants (data are per jar of 3 plants)

	Jar No.	No. of nodules.	Dry wt. of nodules (g.).	Dry wt. of roots (g.).	Dry wt. of tops (g.).	Total dry wt. of plants (g.).	Total N content of plants. (mg.)
Non-aerated	1	114	0·58	0·97	4·70	6·25	153
	2	115	0·59	1·19	5·18	6·96	168
	3	133	0·36	0·84	4·92	6·12	145
	4	220	0·48	0·85	5·22	6·55	157
	Means	**146**	**0·50**	**0·96**	**5·01**	**6·47**	**156**
Aerated	5	259	0·52	1·32	9·03	10·87	297
	6	250	0·74	1·01	8·89	10·64	298
	7	360	0·59	1·08	7·11	8·78	242
	8	265	0·70	1·46	8·64	10·80	297
	Means	**284**	**0·64**	**1·22**	**8·42**	**10·27**	**284**

TABLE III

Harvest Data for 1947 Experiment, Growth Period 15 May–15 August (data are means per jar of 3 plants)

Nodulated Plants

Treat-ment.*	No. of replicate jars.	No. of nodules.	Dry wt. of nodules (g.).	Dry wt. of roots (g.).	Dry wt. of tops (g.).	Total dry wt. of plants (g.).	Total N content of plants (mg.).
S. N.A.	3	205	0·600	0·84	5·64	7·08 (6·6–7·8)†	227
S. A.	3	302	0·695	0·83	6·10	7·63 (6·9–8·1)	245
C. N.A.	3	406	1·362	1·03	13·22	15·61 (14·6–16·2)	495
C. A.	5	655	1·442	1·30	12·95	15·69 (14·7–17·7)	480
			Non-nodulated Plants‡				
S. N.A.	3	—	—	3·38	21·30	24·68 (23·7–25·4)	516
S. A.	6	—	—	3·94	24·91	28·85 (24·4–33·2)	511
C. N.A.	3	—	—	4·22	26·72	30·94 (28·6–32·7)	548
C. A.	3	—	—	3·21	20·00	23·21 (21·7–26·0)	523

* In this column S and C indicate 'supernatant' and 'complete' Crone solution respectively.
† Bracketed figures in this column show the range of variation in the replicate jars.
‡ Supplied with a total of 800 mg. N as sodium nitrate per jar.

growth which was superior in all respects to that obtained in the 'supernatant' solution. Non-nodulated plants receiving nitrate-nitrogen developed equally vigorously in both solutions. In jars containing these latter plants pH showed some fall from the original value of 6·2 during early stages of growth, particularly in the 'supernatant' solution, but later rose to values in the range of 6–7.

With both non-nodulated and nodulated plants the pH in aerated jars was usually higher than in non-aerated jars, the actual difference ranging from 0·1 to 1·0. With nodulated plants in both culture solutions the number of nodules was markedly increased by aeration,[1] without, however, a corresponding increase in nodule dry weight. Aeration had a clearly marked effect on the length and distribution of roots, those in aerated jars extending right to the base of the jar, while in the absence of aeration root and nodule development was chiefly in the upper part of the jar, but in terms of dry weight and nitrogen content of nodulated plants there was little effect. With non-nodulated plants aeration affected the length and distribution of the roots as above, but in respect of plant dry weight no distinct effect can be discerned, having regard to the variation between replicate jars.

The means of 20 oxygen determinations on aerated jars when the plants were 11–12 weeks old were 88 per cent. for the midway level and 93 per cent. for the base. Data for non-aerated jars are presented in Table IV. For nodulated jars the means of all the values are 31 and 21 per cent. for the midway and basal levels respectively, the corresponding means for non-nodulated plants being 58 and 32 per cent.

TABLE IV

*Oxygen Data for 1947 Experiment**

Days from commencement of experiment.	Temperature °C.	Sampling level.	Oxygen content of solution. Jars with nodulated plants.	Oxygen content of solution. Jars with combined N plants.
78	20	Midway	19	48
81	21	Midway	35	60
		Base	18	19
83	19	Midway	39	67
		Base	29	38
84	21	Midway	30	56
		Base	17	38

* All observations were on non-aerated jars with 'complete' solution. Oxygen values are expressed as percentages of equilibrium figures (see text), each value being the mean of observations on 2–3 jars.

1948 experiment. Here the effect of aeration from commercial cylinders was studied on nodulated plants receiving a wide range of cultural treatment (see Table V) in order to determine whether the favourable effect of aeration observed in 1940 could be reproduced under any of the treatments.

The data for non-aerated jars in Table V show that the 'supernatant' solution again proved markedly inferior to the 'complete' solution, while the 'intermediate' solution (with weekly renewal) gave somewhat better growth than the latter solution. In the 'complete' solution, particularly where there

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[1] Since there was little difference in the response to the two sources of air, the data are grouped together.

TABLE V

Harvest Data for 1948 Experiment, all Nodulated Plants, Growth Period 28 April–2 August (data are means per jar of 3 plants, with 3 replicate jars for each treatment)

Treatment.*	No. of nodules.	Dry wt. of nodules (g.).	Dry wt. of roots plus nodules (g.).	Dry wt. of tops (g.).	Total dry wt. of plants (g.).	Total N content of plants (mg.).
C. 7. N.A.	964	1·33	3·22	8·93	12·14 (10·7–13·0)†	347
C. 7. A.	1,400	1·38	3·48	8·66	12·14 (11·3–13·1)	299
C. 14. N.A.	—	—	3·74	8·83	12·57 (10·6–15·3)	327
C. 14. A.	910	1·29	3·39	8·54	11·93 (10·1–13·5)	255
C. pH 14. N.A.	1,060	1·32	3·21	9·97	13·18 (12·5–13·6)	349
C. pH 14. A.	2,165	1·41	3·87	9·53	13·40 (12·5–14·4)	284
Int. 7. N.A.	624	1·71	3·71	10·08	13·79 (12·3–14·8)	373
Int. 7. A.	795	1·05	2·20	6·11	8·31 (7·6–9·5)	239
Int. 14 N.A.	530	1·33	3·13	7·83	10·96 (9·4–11·9)	284
Int. 14. A.	440	1·23	2·65	6·78	9·43 (8·5–10·4)	284
S. 14. N.A.	312	0·83	2·04	4·76	6·80 (6·9–7·3)	—
S. 14. A.	150	0·61	2·03	4·65	6·68 (4·3–8·9)	—

* In this column C., Int., and S. indicate 'complete', 'intermediate', and 'supernatant' Crone solution respectively. The figures 7 and 14 indicate weekly or fortnightly renewal of solution. N.A. and A. signify non-aerated and aerated jars respectively, while 'pH' indicates jars in which pH was restored each day to 6·5.

† Bracketed figures in this column show the range of variation in the replicate jars.

was pH adjustment, aeration was accompanied by a marked increase in the number of nodules, though without any change in nodule or total dry weight, while the nitrogen fixed tended to be lower. In the 'intermediate' solution (particularly with weekly renewal) nodule, root, and top dry weights decreased in the presence of aeration. The non-aerated plants in this solution showed bursting of the outer layers of the tap root and the development of fistular cortex; the nodules on these plants included some relatively very large ones. In the 'supernatant' solution aeration reduced the number of nodules, but in other respects the plants were too variable to permit of comparisons; non-aerated roots showed abnormalities as above. In all solutions aeration again led to a denser growth of roots and more abundant nodules in the lower part of the jars. Observations on plants from the 'complete' solution showed that in non-aerated jars 87 per cent. of the total number of nodules occurred on the upper 10-cm. zone of the roots, while in aerated jars only 33 per cent. were located in that zone, the rest being at lower levels.

Comment on the pH and oxygen data, which are summarized in Tables VI and VII respectively, will be reserved to the Discussion.

TABLE VI

*pH Data for 1948 Experiment (all nodulated plants)**

Cultures with weekly renewal of solution†

Type of jar.‡	Days from commencement of experiment.								
	29.	36.	43.	50.	57.	64.	71.	80.	85.
C. N.A.	6·29	6·10	6·18	5·96	6·04	5·87	5·67	5·28	5·50
C. A.	6·23	6·27	6·23	6·27	6·20	6·12	5·86	5·24	5·50
Int. N.A.	5·78	5·44	5·80	5·52	5·41	4·81	4·39	3·49	3·72
Int. A.	—	5·30	5·89	5·49	5·66	6·00	5·69	4·43	4·49

Cultures with fortnightly renewal of solution§

Type of jar.‡	29.	36.	43.	50.	57.	64.	71.	80.	85.
C. N.A.	5·45		5·89		5·74		5·25		4·76
C. A.	5·33		5·70		5·84		4·97		4·79
Int. N.A.	4.16		4·71		4·52		3·68		3·48
Int. A.	4·23		4·77		4·31		4·18		3·61
S. N.A.	3·82		4·19		4·28		3·91		3·59
S. A.	4·03		4·29		4·25		4·25		4·00

* Each value is a mean for 2–3 jars.

† pH data shown were recorded 3 days after a renewal of solution.

‡ C., Int., and S. signify 'complete', 'intermediate', and 'supernatant' solutions respectively. N.A. and A. signify non-aerated and aerated respectively.

§ pH data shown were recorded 10 days after a renewal of solution.

TABLE VII

*Oxygen Data for 1948 Experiment (all nodulated plants)**

Days from commencement of experiment.	Temperature ° C.	Sampling level.	Oxygen content of solution.	
			Non-aerated jars.	Aerated jars.
30	16	Midway	67	95
		Base	65	94
37	17	Midway	68	96
		Base	66	94
44	20	Midway	40	96
		Base	42	89
63	22	Midway	15	74
		Base	1	64
75	14	Midway	44	92
		Base	43	90
83	21	Midway	31	77
		Base	21	70

* All observations were on jars with 'complete' solution. Oxygen values are expressed as percentages of equilibrium figures (see text), each value being the mean of observations on 2–5 jars.

DISCUSSION

1. *Growth in different culture solutions*

In the above experiments the following versions of Crone's solution have been employed (see p. 247 for explanation of terms):

(*a*) 'Complete' Crone,
(*b*) as (*a*) but with daily adjustment to pH 6·5,
(*c*) 'Intermediate' Crone,
(*d*) 'Supernatant' Crone,
(*e*) as (*d*) but with daily adjustment to pH 6·5.

Considering nodulated plants first (non-aerated jars), solutions (*a*) and (*b*) both gave plants of healthy appearance and satisfactory fixation of nitrogen, the dry weight of plants in solution (*b*) being slightly superior (1948 experiment). Solution (*c*) in 1948 gave nodulated plants of slightly greater dry weight and nitrogen fixation than solutions (*a*) or (*b*), other features being that nodules were less frequent but of relatively large size, while the roots showed some abnormalities (p. 252). The generally favourable nature of this solution was a matter for surprise, since the addition of insoluble sediment was only a small one, and also the solution is of low buffering power, leading to the prevalence of relatively low pH. According to Bryan (1922) a pH of 5 or less is harmful to nodulated soya-bean plants if applied from the commencement of growth. As shown in Table VI, in jars receiving weekly renewal pH in solution (*c*) remained for the most part in the range 5–6 during the early weeks of growth in which most of the nodules were initiated. Subsequently, over the period in which most of the growth and fixation of nitrogen took place, pH was for a considerable part of the time below 4·5 and even below 4·0. These observations agree with those of Jensen (1943), who reported for *Medicago* and *Trifolium* that though the number of nodules formed is highest at a reaction near to neutrality, nodules once formed may continue to fix nitrogen at pH which is so low as to prohibit new nodule formation.

The experiments of 1947–8 with solutions (*d*) and (*e*) show that the soluble portion alone of the Crone medium does not support healthy growth of nodulated soya-bean plants in experiments of long duration.[1] Solution (*d*) is characterized by unstable pH in contact with nodulated plants, so that values in the region of pH 4 prevailed for a considerable part of the experiment (Table VI), but the fact that (*e*) in 1947 gave no better results suggests that pH is not mainly responsible. It is likely that the solution will be deficient in phosphorus, calcium, and iron on the basis of commonly accepted minimal requirements for culture solutions, while at the same time there is a large excess of potassium and chloride. Comparison of growth in solutions (*d*) and (*e*) with that in the solutions containing insoluble constituents indicates that the sediment is definitely of value to the plants (Marais, 1922; Miller, 1938, p. 244).

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[1] It is concluded that in the 1940 experiments, in which good growth was obtained in what was nominally 'supernatant' solution, sufficient sediment was included to prevent the deficiencies which arise when the solution is completely free of sediment.

Only solutions (*a*) and (*d*) have been employed with non-nodulated plants supplied with nitrate. In the direct comparison provided by the 1947 experiment, growth in both solutions was strong, that in (*d*) being not definitely inferior to growth in (*a*); pH changes were relatively slight with both solutions. Thus we have a solution which fails to support good growth of nodulated plants but which is satisfactory with non-nodulated plants receiving combined nitrogen, suggesting that the mineral requirements of the two types of plant differ. The scanty information on this question has been reviewed by Wilson (1940).

2. *Further analysis of pH drifts*

The main findings are: (1) the pH of solution in contact with nodulated plants falls with some rapidity, varying with the different versions of the Crone solution and with the stage of plant development; (2) with nodule-free plants supplied with nitrate there is no corresponding fall, and in later stages pH of the solution increases after contact with the roots of the plants; (3) with both types of plant pH in aerated jars is usually, though not always, higher than in non-aerated jars.

As regards (1) and (2) the possibility arose that the tendency for a depression of pH exists also with non-nodulated plants, but that it is counterbalanced by the well-known opposite tendency for the presence of nitrate as the sole source of nitrogen to lead to an increase in pH. That this is actually the case is indicated by tests carried out in 1948 on non-nodulated plants normally supplied with nitrate, specially grown for the purpose. In the jars of such plants, temporarily deprived of nitrate, a fall in pH from 6·2 to 4·5 was shown in 4 days, while in corresponding jars with nitrate present pH remained in the range 5·9–6·1.

This diminution in pH shown by Crone's solution in contact with nodulated and non-nodulated plants of soya-bean is no doubt due in part to accumulation of carbon dioxide, but the main factor is presumably the unequal absorption of the ions of the salts of the nutrient solution.

3. *Effect of aeration. Oxygen relation*

Aeration may tend to change conditions in the culture jars by preventing the depletion of oxygen in the solution, and also that of nitrogen where there is any utilization of that gas, as in fixation. The removal of respiratory carbon dioxide will be accelerated, which may in turn result in pH in aerated jars being somewhat higher than in those without aeration. Such differences in pH tended to exist in the present experiments, but for the most part were relatively small. In addition the air-stream will have stirring effects, which might, for example, result in the nodule bacteria being spread more effectively over the root system, giving greater infection, though this is not necessarily beneficial to the plants.

It will be assumed that, of the above, the oxygen factor is likely to be of chief importance. It is then necessary to consider the extent of the differences in dissolved oxygen between aerated and non-aerated jars with the arrangement employed in the present experiments, which allowed fairly free access of atmospheric oxygen to the surface of the culture solution in non-aerated jars. The data presented show that during the first half of the growth period oxygen tension of 99–100 per cent. of equilibrium values were maintained in aerated jars, tensions recorded later being mostly upwards of 75 per cent. of equilibrium, although some lower values were obtained. With non-aerated jars of nodulated plants, values in the region of 65 per cent. were obtained midway down the jars with plants 4–5 weeks old, after which tensions fell to somewhere in the range of 15–45 per cent. of equilibrium. With non-aerated jars of combined nitrogen plants the reduction in oxygen tensions was less marked. It was clear that oxygen content was closely dependent on temperature, being much lower on relatively warm days, no doubt as a result of the greater effect of increased temperature on root respiration than on the rate of diffusion of fresh oxygen into the solution. Oxygen content at the base of the non-aerated jars was usually lower than that at the midway level, and values at or near to zero were sometimes obtained for the former region (see also later).

It is clear from the above that the use of unsealed cultures and the method of depending on the activity of the plants to produce a deficiency of oxygen result in poor control over oxygen content. The extent of the oxygen deficiency is not steady during the experiment, but increases as the plants become larger and to a different degree in jars containing nodulated and non-nodulated plants respectively. No determinations were carried out on the solution above the midway level, but it may be assumed that oxygen tensions there in non-aerated jars were higher than those at midway, and that at least in the earlier stages of the experiments little difference existed at these higher levels between aerated and non-aerated jars.

The differences in growth and activity actually observed between plants of aerated and non-aerated jars in these experiments have been indicated in the previous section and, for nodulated plants, are obviously too discrepant to attempt at this stage any unified treatment or final conclusions. The following observations may, however, be made.

1. In the 1940 experiments aeration resulted in all-round benefit to nodulated plants, i.e. the result resembled that obtained by Virtanen and von Hausen (1936; see Introduction to the present paper) for peas with an essentially similar experimental arrangement. In later trials with varied experimental procedure no definite benefit from aeration to nodulated plants was observed and in some respects it was detrimental. An explanation of these divergent results cannot be offered at present. The nature of the result seems to be affected by the type of culture solution employed, and possibly by climatic and other factors not at present appreciated.

2. The sparsity of roots and nodules in the lower regions of non-aerated

jars suggests that oxygen tension there was limiting, but the ability of the roots and nodules to develop relatively freely in the higher oxygen tensions prevailing in the upper parts of the jars, both with nodulated and non-nodulated plants, means that a clear demonstration of the relative importance of oxygen to the two types of plants is not necessarily given by the experimental arrangement used in the present work, since taking the jar as a whole critical oxygen tensions may not be attained. The possibility of variation in result from one experiment to another exists, since under conditions conducive to rapid growth and exhaustion of oxygen, limiting values of the latter may be approached to a greater extent than when growth is slower. Experiments with an improved arrangement, resembling that employed by Gilbert and Shive (1942) and giving closer control over oxygen tension, are in progress.

3. Bearing in mind the marked depletion of oxygen recorded for the midway level in the non-aerated culture jars during the later periods of the 1947/8 experiments, and that an appreciable proportion of the roots and nodules occur in this region, the data presented suggest that roots (of both nodulated and non-nodulated plants) and nodules are able to function without serious diminution of efficiency under appreciably reduced oxygen tension, since dry weights and nitrogen fixation for non-aerated plants were not lower than for aerated plants.

4. It is felt that the increase in the number of nodules observed in most instances as a result of aeration may be due to a stirring effect, since during the earlier stages of the experiments when nodule initiation was most active the differences in oxygen tension between aerated and non-aerated jars were not marked.

5. The results obtained with combined-nitrogen plants may be compared with those of Gilbert and Shive (1942), who, using sealed constant-flow cultures of non-nodulated plants of soya-bean supplied with nitrate-nitrogen, studied the effects of passing nitrogen/oxygen gas mixtures containing respectively 40, 20, 10, and 0 per cent. oxygen for a period of 23 days. Although symptoms ascribed to oxygen toxicity were observed at 40 and 20 per cent., being apparently confined to the tops, differences in dry weight were not very marked except at zero oxygen, and taking dry weight at 40 per cent. oxygen as 100, the relative figures for the progressively lower proportions of oxygen were 94, 95, and 79. In the present experiments oxygen tensions recorded midway down non-aerated jars of combined-nitrogen plants in later stages of growth were of the order of 50 per cent. of equilibrium, equal to that produced by passing a nitrogen/oxygen mixture containing 10 per cent. oxygen. Higher tensions no doubt prevailed at earlier stages, and also higher up the jars. That there should be little difference in growth between plants in such jars and those in jars receiving aeration is in general agreement with the results of Gilbert and Shive.

6. Allison and Shive (1923) examined the response to aeration of non-nodulated soya-bean plants in water culture (plus nitrate). In static cultures aeration resulted in greater root length and slightly increased root dry weight,

but the dry weight of the whole plants was slightly less than in non-aerated jars. With constant-flow cultures, on the other hand, aeration led to considerably increased dry weight. The authors suggested that some growth-retarding factor (the development of a somewhat unfavourable pH is mentioned) prevented a response to aeration in static cultures, in which growth, besides being smaller than with constant flow, was somewhat abnormal. In most of the experiments of the present paper healthy growth of plants was obtained, with no suggestion of abnormality or inhibition of growth in non-nodulated or nodulated plants. It may also be noted that increasing the frequency of renewal of the 'complete' solution from fortnightly to weekly in 1948 did not lead to greater growth.

7. Gilbert and Shive (1945) concluded that soya-bean and other plants (tomato, oat) have the ability to carry on a special type of anaerobic respiration in which the nitrate ion is used as a source of oxygen. Their results indicate that this nitrate respiration is at a maximum in zero oxygen, and that when the culture solution is maintained at 4 p.p.m. oxygen (equal to 50 per cent. of value for water in equilibrium with air) nitrate respiration falls to $\frac{1}{8}$th of its maximum, presumably signifying that the plants are now making use chiefly of ordinary aerobic respiration. It thus appears that at the oxygen tensions prevailing in the non-aerated jars of combined-nitrogen plants in the present experiments, nitrate respiration was of little importance, and was unlikely to impart to those plants any special immunity to the effects of low oxygen tension not shared by the nodulated plants. In future experiments it will, however, be desirable to include plants supplied with ammonia-nitrogen in order to test the matter fully.

4. *Further observations on the rate of depletion of oxygen in non-aerated jars*

As noted in the 1947 experiment, oxygen tensions in non-aerated jars of nodulated plants were consistently below those in corresponding jars of combined-nitrogen plants. This suggests a greater rate of oxygen consumption by nodulated as compared with non-nodulated roots on a per plant basis, since opportunities for replenishment of oxygen by diffusion appear to have been at least as good in jars of nodulated plants as in those of non-nodulated plants.

In order to gain further data an experiment was carried out on nodulated plants and non-nodulated plants receiving nitrate (2 jars of each) from the 1947 experiment, when the plants were 12 weeks old. The jars were filled with fresh solution, and then by aeration the oxygen content was brought close to the equilibrium figure, when air-currents were stopped. A control jar with no plants was included. Changes in oxygen tension at midway and basal levels in the jars were followed at intervals during hours of daylight over a total period of 44 hours, during which day-time temperatures in the greenhouse ranged from 19° to 23° C. Table VIII shows that from the 5th hour onwards, oxygen tensions in the jars of nodulated plants were notably below those for the non-nodulated plants. By the 28th hour oxygen tension at the

TABLE VIII

Data of Experiment on Rate of Depletion of Oxygen

	Sampling level.	Oxygen content of solution*					
		Start.	1½ hours.†	5 hours.	20 hours.	28 hours.	44 hours.
Jars with nodulated plants‡	Midway	97	75	42	32	14	25
	Base	93	70	35	8	0	0
Jars with combined nitrogen-plants‡	Midway	96	80	51	64	47	48
	Base	94	69	33	45	21	23
Control jar, no plants	Midway	101	99	97	96	92	91
	Base	97	101	—	87	88	83

* Expressed as percentage of equilibrium figure (see text).

† Hours from start of experiment.

‡ Oxygen values for jars with plants are means of 2 jars.

base of the jars of nodulated plants was actually zero. The apparent fall in oxygen tension in the control jar is probably due to temperatures being somewhat lower in the later part of the experiment.[1] The mean dry weights for the plants used in this test were, at harvest shortly afterwards, as follows:

	Roots.	Nodules.	Total
Nodulated plants	1·04 g.	1·33 g.	2·37 g.
Non-nodulated plants plus nitrate .	4·41 g.	—	4·41 g.

These data support the conclusion that oxygen utilization by nodulated roots is considerably greater than by non-nodulated ones. Assuming respiratory quotients not greatly in excess of unity, the above conclusion is in keeping with that of previous experiments on plants grown under similar conditions (Bond, 1941; see Introduction to present paper) which indicated that the evolution of carbon dioxide per unit dry weight of soya-bean nodule tissues was considerably greater than for root tissues.

SUMMARY

1. Observations are presented on the growth in static, non-flowing water culture of nodulated plants of soya-bean in nitrogen-free solution, and of corresponding non-nodulated plants supplied with nitrate-nitrogen.

2. Satisfactory growth and fixation of nitrogen were obtained with nodulated plants in Crone's solution containing the full complement or a proportion of the insoluble constituents, but not in a medium comprising the soluble portion only. The insoluble constituents thus appear to be of value to the plants. Non-nodulated plants supplied with nitrate-nitrogen made satisfactory growth in the medium with soluble material only, suggesting a difference in mineral requirements between the two types of plant.

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[1] The method of expression of results of oxygen determinations adopted in this paper assumes instant re-establishment of equilibrium with the atmosphere when the temperature of the solution changes. Actually equilibrium will only be regained after an interval of time, and because of this the method of expression introduces small errors in respect of determinations made under conditions of fluctuating temperature.

3. The pH of Crone's solution in contact with nodulated roots fell from the initial value of 6·2 with some rapidity, varying with the different modifications of the solution that were employed. With nodule-free plants supplied with nitrate-nitrogen a corresponding fall in pH was not shown, but a fall did occur if such plants were temporarily deprived of nitrate.

4. Evidence was obtained that, after the period of maximum formation of nodules is passed, nodulated plants grow well at relatively low pH (3·5–5·0).

5. Well-marked differences in the oxygen content of the solutions midway down and at the base of the culture jars were shown between non-aerated and aerated jars, particularly in the later stages of growth. Differences were probably smaller in respect of the solution in the upper part of the jars, since the arrangement employed allowed fairly free access of atmospheric oxygen to the surface of the culture solution.

6. In the experiments of the first year aeration led to all-round benefit to nodulated plants, but in subsequent experiments under a wide range of cultural treatments, some of which appeared to reproduce closely the conditions of the first year, aeration led in most cases to greater root length and to more numerous nodules, but little or no benefit ensued in terms of dry weight or nitrogen fixation, which were sometimes better in non-aerated jars. Non-nodulated plants receiving nitrate-nitrogen grew equally vigorously with or without aeration in all instances.

7. No adequate explanation of these discrepancies with nodulated plants can be offered at present. In any case it is felt that for reliable data on the relative importance of oxygen to nodulated and non-nodulated roots it is essential to adopt a method giving closer control over the oxygen content of the culture solution. Experiments with an improved method are in progress.

8. The results do permit the conclusion that roots (of both nodulated and non-nodulated plants) and nodules are able to function efficiently at appreciably reduced oxygen tension.

9. In the absence of aeration the oxygen content of jars containing nodulated plants was usually less than that in jars with non-nodulated plants supplied with combined nitrogen, despite the greater mass of the roots of the latter plants. A more rapid intake of oxygen per unit dry weight of nodulated root is suggested, though it does not necessarily follow from this that such roots will be specially sensitive to reduced oxygen supply.

Dr. J. Boyes carried out preliminary experiments in connexion with the work described in this paper, and was responsible for several features of the experimental arrangement. A grant from the Agricultural Research Council has facilitated the investigation, in which technical assistance has been given by Miss M. P. McGonagle, and Messrs. T. P. Ferguson and J. C. Leitch. The author is indebted to Dr. S. Williams for his criticism of the text of the paper.

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Fungal Parasites of the Phytoplankton. I

(Studies on British Chytrids, X)

BY

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With one Plate and sixteen Figures in the Text

THE planktonic algae of four bodies of water in the English Lake District (i.e. Windermere, North and South Basins, Blelham Tarn, and Esthwaite Water) have been examined at weekly intervals from October 1946 to the time of writing, April 1949, for the presence of aquatic fungi. A few records of fungi prior to October 1946 are also included. The more important algae present in these lakes belong to the classes Chlorophyceae, Bacillariophyceae, Chrysophyceae, Xanthophyceae, Myxophyceae, Dinophyceae, and Cryptomonadineae. It is only on the latter class that no fungus, saprophytic or parasitic, has been observed. By far the most abundant fungi belong to the Uniflagellatae series of Phycomycetes, but a few Biflagellatae forms have been observed. Altogether thirty different organisms are now known, three being saprophytic and the rest parasitic. The parasitic fungi sometimes increase rapidly, reaching epidemic proportions, and in consequence a particular species of alga may have its numbers severely reduced. This has already been demonstrated (Canter and Lund, 1948) for the diatom *Asterionella formosa* Hass. which is parasitized by *Rhizophidium planktonicum* Canter. Other similar investigations are being carried out on *Fragilaria crotonensis* (A. M. Edw.) Kitton, *Melosira italica* (Ehrenb.) Kütz., *Sphaerocystis Schroeteri* Chod., *Gemellicystis neglecta* (Teiling) Skuja, and the many desmids which occur in the plankton.

This paper deals with the life-histories of a few of the more abundant chytrids. Many others have been noted, but the knowledge of their structure and life-cycles is not sufficient to warrant description at this stage.

In 1949 an investigation of phytoplankton parasites from seventeen other lakes in the English Lake District was started. At the time of writing this survey is only a few months old, but already many of the parasites common to Windermere, Esthwaite Water, and Blelham Tarn have been found, and records of their occurrence are given in the relevant tables.

I. *RHIZIDIUM WINDERMERENSE.* N. SP.

Gemellicystis is a colonial green alga surrounded by a wide mucilage sheath (Fig. 1, A) to which may be attached cells of *Chlamydomonas epiphytica*

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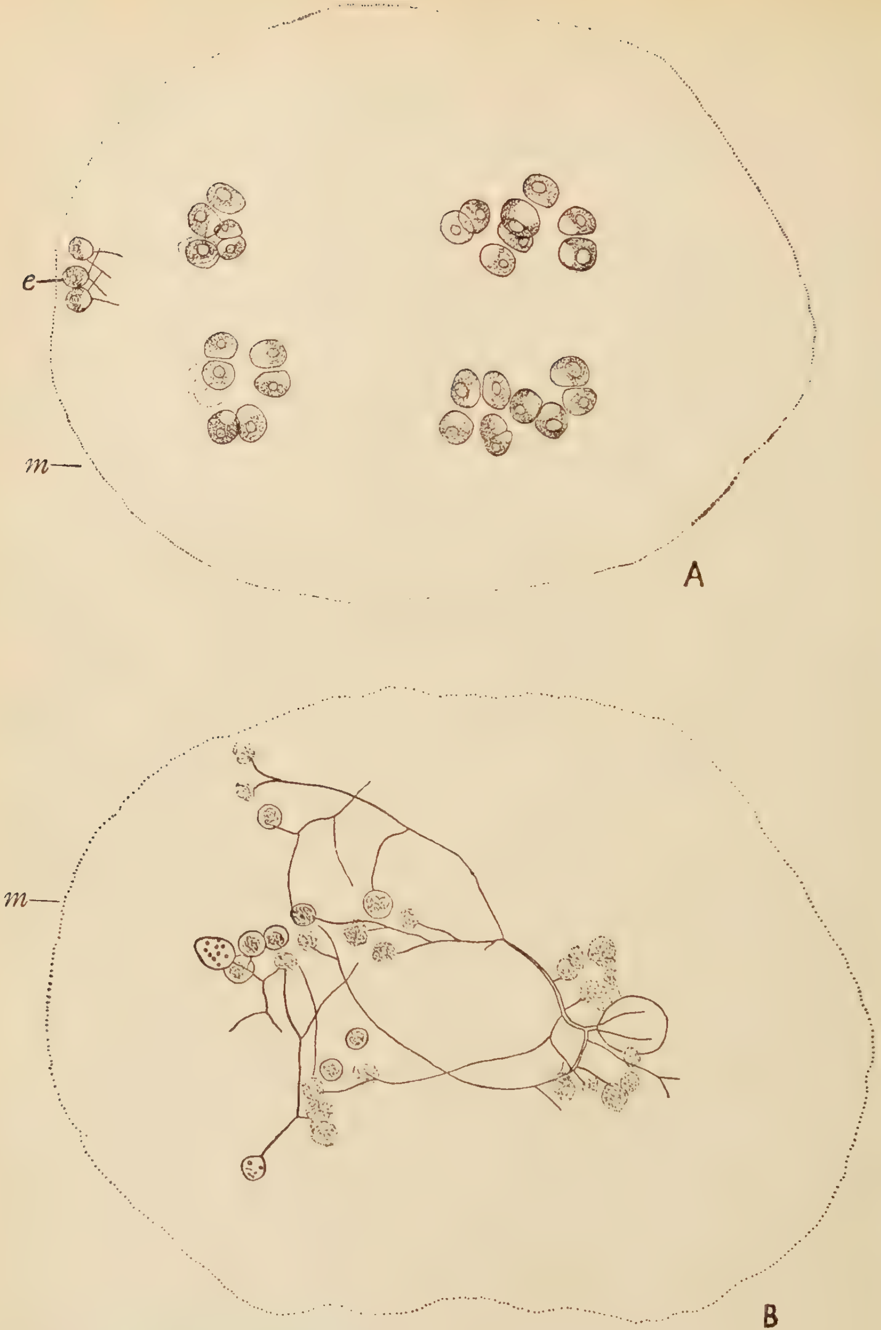

FIG. 1, A, B. *Rhizidium windermerense* n. sp. A, a healthy colony of *Gemellicystis neglecta* with three cells of the epiphyte *Chlamydomonas epiphytica*. Drawn by Dr. J. W. G. Lund. B, colony infected by *Rhizidium windermerense*. m, mucilage. (A, × 520; B, × 470.)

G. M. Smith (Fig. 1, A, *e*). The parasite is polyphagous and a single thallus may kill nearly all the cells in a host colony (Fig. 1, B). The content of the diseased cells shrinks and aggregates, and, in the advanced stages of infection, the cell walls tend to disintegrate.

TABLE I

Occurrence of R. windermerense *on* Gemellicystis neglecta (*Teiling*) *Skuja*

Lake.	1947.	1948.	1949.
Windermere: North Basin	June 15–Nov. 17	Aug. 30–Oct. 11	Apr. 19–May 9
Windermere: South Basin	June 11–Aug. 5, Oct. 14	Sept. 8–Oct. 26	Apr. 28–May 10
Blelham Tarn	Oct. 1–13	—	—
Esthwaite Water	June 13–July 10	July 12–23	—
Derwent Water	—	—	Feb. 10–Mar. 24, Apr. 7

The zoospore of *Rhizidium windermerense*, having penetrated for some distance into the mucilage surrounding the alga, gives rise to an unbranched or little-branched rhizoid which grows towards, and ultimately makes contact with, a host cell (Fig. 2, A). A lateral branch often develops close to the sporangial rudiment and grows in approximately the opposite direction to that of the original germ-tube so that the thallus may have a rhizoidal system, as shown in Fig. 2, I.

The rhizoids are long and taper towards their extremities. Fine branches pass to the individual host cells, but, owing to the dense content of the latter, the internal rhizoidal system, which presumably exists, has not been observed. Other fine branches penetrate through the mucilage (Fig. 4). The encysted zoospore enlarges directly into the sporangium. When young this is spherical (Fig. 2, B), but later a thin-walled apical papilla develops so that the mature sporangium is somewhat pear-shaped (Fig. 2, G). The sporangia vary from 8μ high by 6μ broad to 26μ high by 23μ broad and contain from 5 to 60 or more zoospores according to their size. Dehiscence has been observed only a few times, but there can be no doubt that this fungus is inoperculate. The zoospores emerge in a mass (Fig. 2, H) and are finally differentiated outside the sporangium. Each is spherical with a large oil globule and single posterior flagellum.

Towards the end of an epidemic caused by this chytrid sexually formed resting spores appear. Several may occur in a host colony, alone or mixed with asexual sporangia (Fig. 4).

Two spherical bodies (resembling encysted zoospores) which I have regarded as sexual thalli give rise to fine threads ('conjugation tubes'). These apparently meet at their tips. The content then passes from the sexual thalli along the conjugation tubes and a swelling is formed at their junction. This swelling (Fig. 3, A–D) is the incipient resting spore or zygote. At this stage

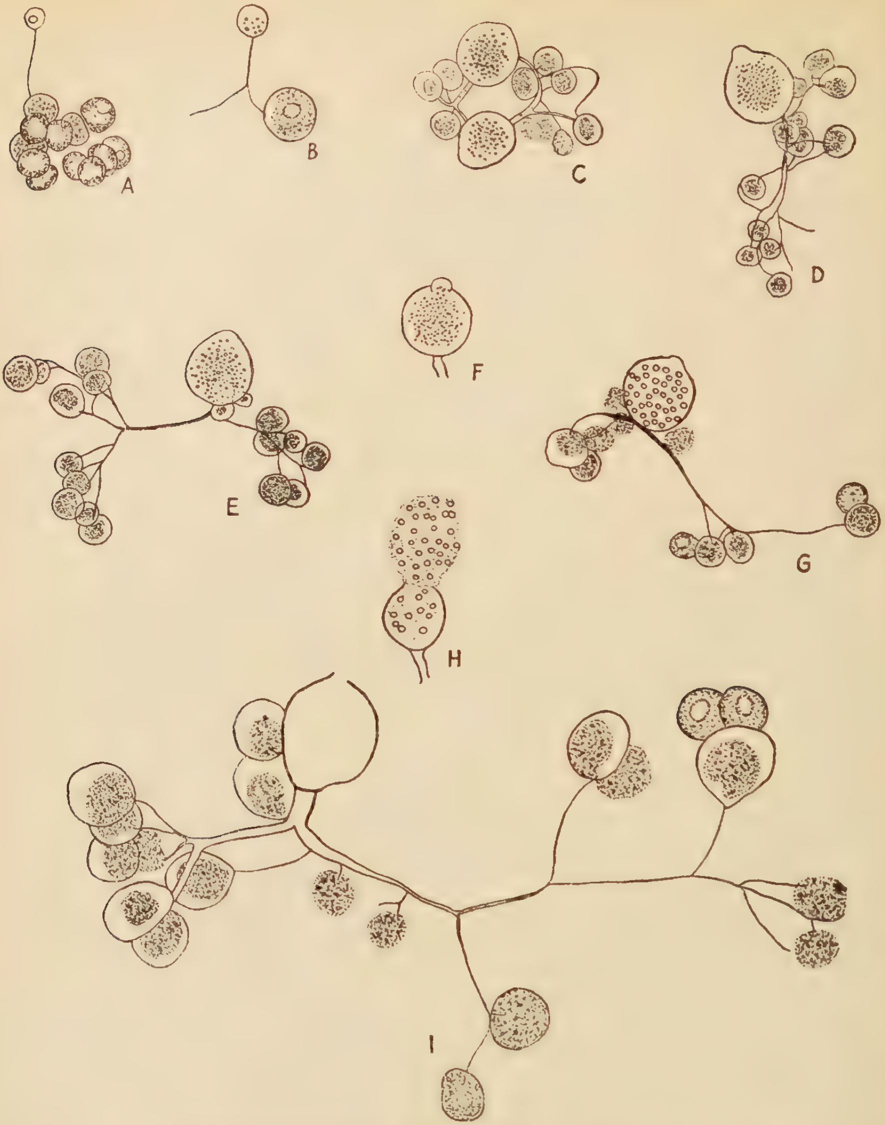

FIG. 2, A–I. *Rhizidium windermerense* n. sp. A, encysted zoospore with germ-tube. B–F, stages in the development of the sporangium, and growth of the rhizoidal system. G, mature, sporangium with dehiscence papilla. H, content emerging from the sporangium on dehiscence. I, empty sporangium with broad rhizoidal axes which taper towards their extremities. (A–F, H, × 500; B, I, × 850; G, × 512.)

the protoplasm is homogeneous and it is surrounded by a thin, colourless wall. There is apparently no constant difference in size between the fusing thalli. The empty sexual thalli vary from 2·6 to 5·9μ in diameter. At an early stage, perhaps even before the content passes out of the thalli, a few fine branches are formed on the conjugation tubes which may pass to a host cell

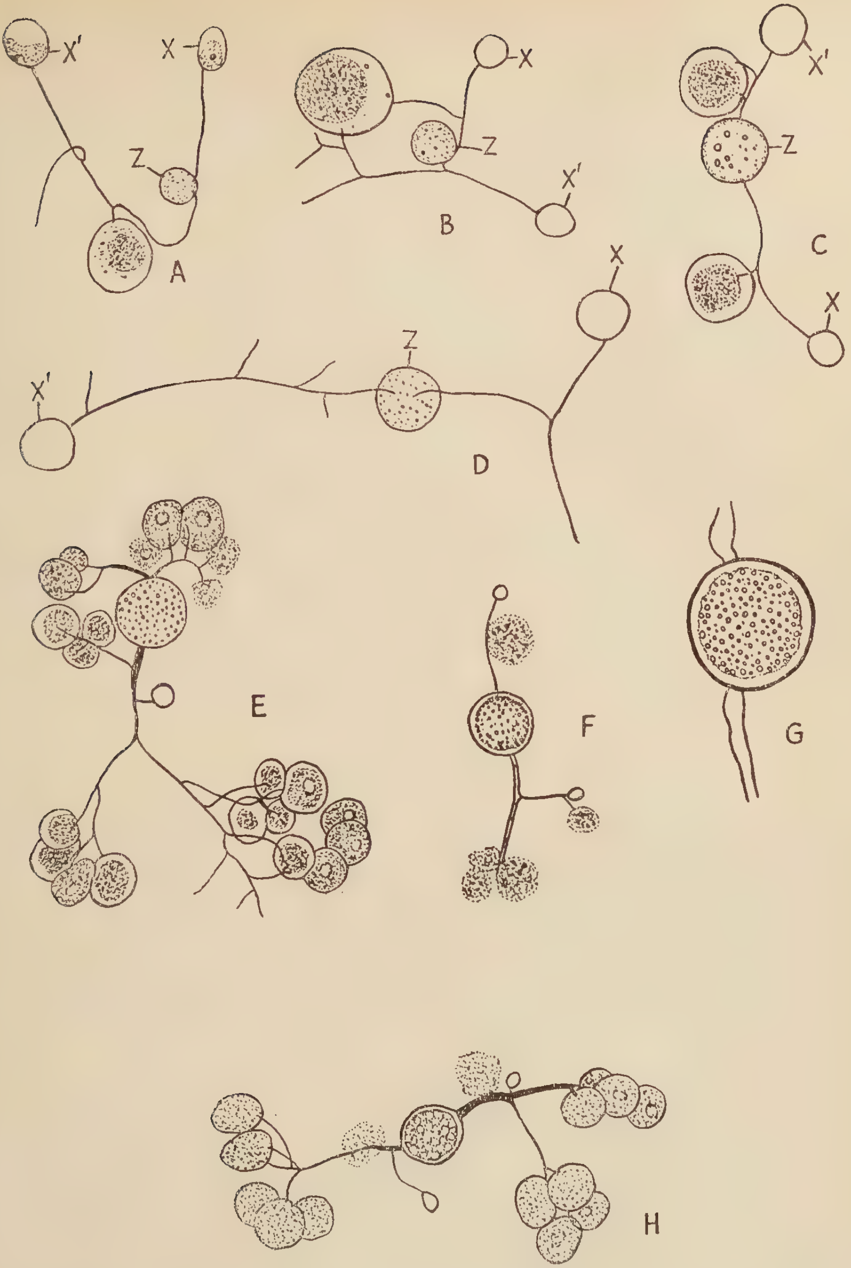

FIG. 3, A–H. *Rhizidium windermerense* n. sp. A, very early stage in fusion, content of associating gametes not yet wholly emptied. B–F, stages in growth of the resting spore; E, shows extensive rhizoidal system, and F the thickening of the zygospore wall. G, mature zygospore, with greatly thickened conjugation tubes, which now form a part of the rhizoidal system. H, mature resting spore and its rhizoidal system.

In figs. A–D: associating gametes, X, X$^{\mathrm{I}}$; young resting spore, Z. (A–E, G, × 1130; F, × 670; H, × 800.)

or into the mucilage (Fig. 3, A, B). However, after protoplasmic fusion has taken place a large rhizoidal system is formed which resembles in its nature, and almost in extent, that of the sporangium (Fig. 3, E).

When mature the zygote is more or less spherical (14–17μ). It has a thick, smooth wall which may become yellowish in colour, and the content consists of numerous small globules (Fig. 3, H).

In its polyphagus habit this fungus resembles species of *Rhizophlyctis* and *Polyphagus*. However, it differs from the latter in the zoospore enlarging directly into the sporangium. Although similar in development to species of *Rhizophlyctis* it again differs from this genus by virtue of its single-branched rhizoidal axis arising from the sporangium. This feature characterizes the genus *Rhizidium*, into which the writer places this fungus for the time being as a new species, *R. windermerense*. The method of formation of the resting spore is unlike any other chytrid, although it is not so far remote from that described by Sorokin (see Sparrow, 1943, p. 389) for *Zygochytrium aurantiacum*. However, it is presumably not monoecious, and although germination of the resting spore has not been observed, it seems unlikely that it produces a hypha-like structure as recorded for *Zygochytrium*.

In its sexual process it resembles *Polyphagus* and more especially *P. Euglenae* Nowak. sense nov. Bartsch, where the resting spore develops subterminally (see Bartsch, 1945, figs. 9, 12). However, in this genus the conjugating thalli have already grown to a considerable size before fusion takes place.

Rhizidium windermerense sp. nov.

Thalli monocentrici, eucarpici, polyphagi, sporangiis et rhizoideis principalibus et ramulis secundariis muniti. *Sporangium* inoperculatum piriforme, 8μ altum × 6μ latum–26μ altum × 23μ latum zoosporis 5–60, globosis, uniguttulatis, postice uniflagellatis. *Sporae* perdurantes (zygosporae) globosae vel subglobosae multiguttulatae, 10μ diam., membrana crassa levi, ex conjunctione apicali rhizoideorum principalium thallorum duorum parvorum formatae. Hab.: In cellulis vivis *Gemellicystidiis neglectae* Windermere, Esthwaite Water, Blelham Tarn et Derwent Water, Anglia.

Rhizidium windermerense n. sp.

Thallus monocentric, eucarpic, polyphagous, consisting of a sporangium (the body of the encysted zoospore) and a single main rhizoidal axis which by the development of secondary branches loses its original taproot-like appearance. Sporangium inoperculate, pear-shaped, 8μ high by 6μ broad to 26μ high by 23μ broad, containing from 5 to over 60 zoospores. Zoospores spherical, uniguttulate and uniflagellate, emerging in an undifferentiated mass. Zygospore spherical to subspherical, 14–17μ in diameter, wall thick, smooth, sometimes yellowish, content consisting of numerous small globules; formed after conjugation of the tips of the rhizoids from two thalli, the contents of both passing out to form a swelling, the incipient resting spore at their point of fusion. Rhizoidal system as in the sporangium developing after fusion of

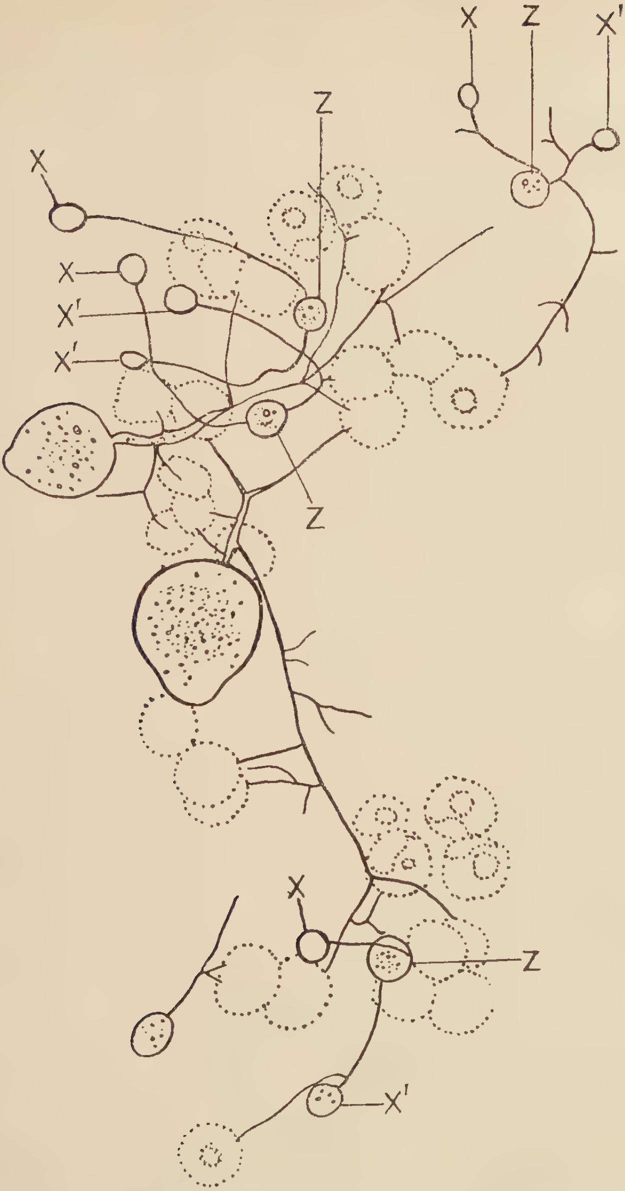

FIG. 4. *Rhizidium windermerense* n. sp. A *Gemellicystis* colony containing three zoosporangia and several very young zygospores. Associating gametes (x, x^{I}), zygospore (z). The contents of the host cells are omitted for simplicity. (× 1025.)

the sexual thalli. The latter remain as empty appendages ($2\cdot6$–$5\cdot9\,\mu$ diam.) on the rhizoidal system of the mature spore.

Parasitic on *Gemellicystis neglecta* (Teiling) Skuja from Windermere North and South Basins, Esthwaite Water, Blelham Tarn, and Derwent Water, in the English Lake District.

II. *RHIZOPHIDIUM EPHIPPIUM* N. SP.

TABLE II

Occurrence of R. ephippium *on* Stylosphaeridium stipitatum *Geitler*

Lake.	1948.
Windermere: North Basin .	July 19–Dec. 13
Windermere: South Basin .	July 14–Nov. 10
Esthwaite Water . .	Oct. 12

The zoospore, having passed through a part of the mucilage surrounding a *Coelosphaerium* colony, settles on the broad upper surface of a *Stylosphaeridium* cell[1] (Fig. 6, A). It swells and then enlarges laterally so that the young sporangium is rather bean-shaped and arches over the host cell (Fig. 6, F, G). As the sporangium matures, the sides become drawn out to form two lateral papillae (Fig. 6, K, L, M). The protoplasm never contains many oil globules

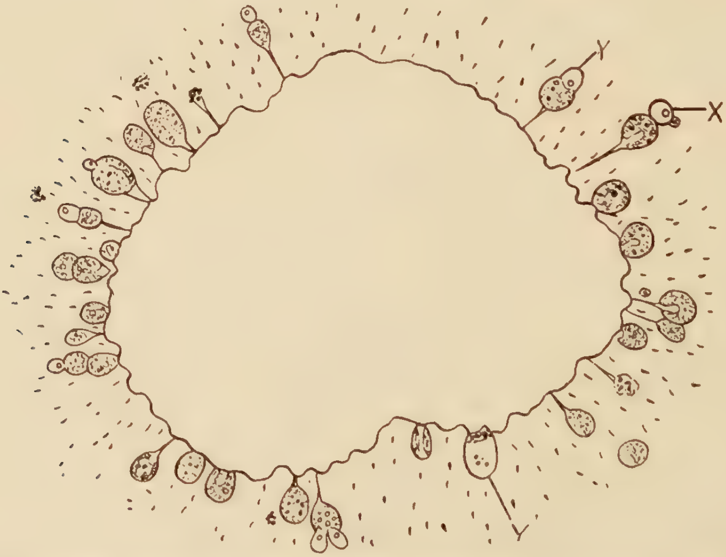

FIG. 5. A general picture of *Stylosphaeridium stipitatum* epiphytic on *Coelosphaerium Naegelianum*. A young resting spore of *Rhizophidium ephippium* is shown at (X), the other chytridiaceous bodies (Y) belong to a second parasite whose life-history is incompletely known. The broken lines indicate bacteria embedded in the mucilage of the *Coelosphaerium*. (× 683.)

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[1] *Stylosphaeridium stipitatum* is an epiphyte on the colonial blue-green alga *Coelosphaerium Naegelianum* Kütz.

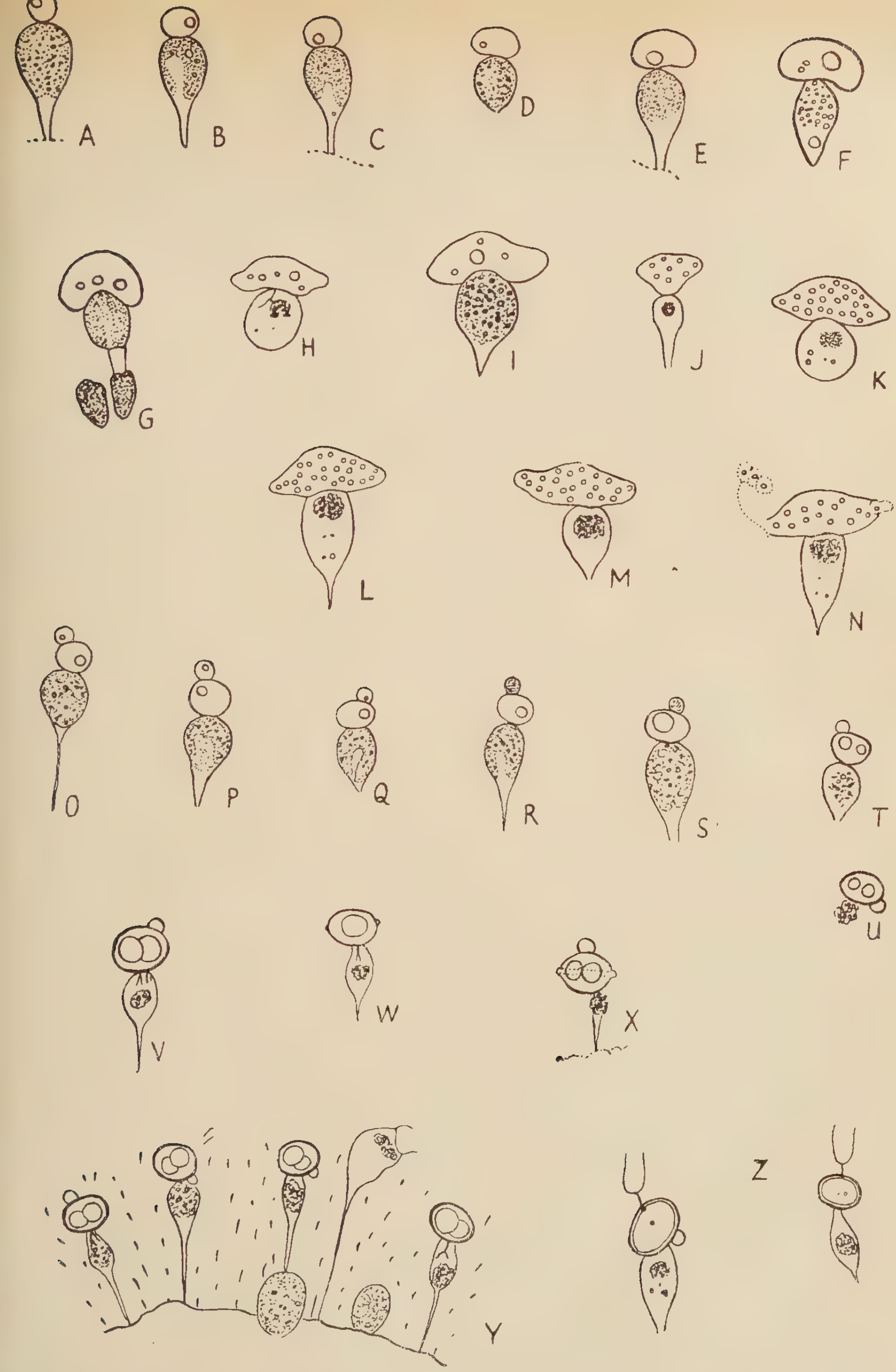

FIG. 6, A–Z. *Rhizophidium ephippium* n. sp. A–I, stages in development from the encysted zoospore to the immature sporangium. J–M, mature sporangia. N, dehisced sporangium with zoospores. O–Q, female cells with recently attached male thalli. R, S, male thalli in which the refractive globule has disappeared but the protoplasmic contents have not completely passed into the female cells. T, empty male cell, wall of female not yet thickened. U–X, mature zygotes; W, viewed from the end, X, shows central girdle. Y, a portion of the mucilage surrounding a *Coelosphaerium* colony (embedded in which are bacteria), showing four *Stylosphaeridium* cells with mature fungal zygotes. Z, two zygotes bearing empty sporangia of a hyperparasite. (A–Z, × 1066.)

during the growth of the sporangium, but at maturity there are from 8 to 30 equal-sized globules each indicating the position of a zoospore. The mature sporangium varies from 8 μ broad by 4 μ high to 15 μ broad by 5 μ high. The majority of the sporangia possess two dehiscence papillae, but occasionally only one is present. On dehiscence the papillae dissolve and the zoospores squeeze their way singly through the openings (Fig. 6, N), passing through the mucilage sheath of the *Coelosphaerium* colony to reach the water. The zoospore is spherical (2 μ) with a small refractive globule and single posterior flagellum. The sporangium wall is very delicate and apparently soon disappears after dehiscence, for only a few empty ones have been observed.

The host chloroplast is always reduced to a mass of reddish granules which are often located immediately beneath the sporangium (Fig. 6, J–N), leaving the remainder of the cell empty. No rhizoids have been seen in this empty region; therefore it is reasonable to suppose that they are not extensive, and in most instances must be buried in the host residue. Only once have I observed any structure resembling a rhizoid connected with a sporangium (Fig. 6, H).

Resting spores are formed by a sexual process identical with that already described for *Rhizophidium goniosporum* Scherffel (1925), *R. fallax* Scherffel, and *R. planktonicum* Canter (1948). The earliest stages in this process are shown in Fig. 6, O–S. The male (essentially an encysted zoospore) attaches itself to a spherical female cell (resembling a slightly enlarged zoospore). The oil globule of the male diminishes in size and finally disappears, so that its protoplasm appears to be evenly granular. This protoplasm must eventually pass into the female, for the male cell adherent to each mature zygote is always devoid of contents (Fig. 6, T, U). The female thallus enlarges after a male has made contact with it, and at maturity it is broadly oval to subspherical in shape, 5 μ broad by 3·7 μ high to 7 μ broad by 5 μ high (Fig. 6, V, Y). The contents consist of two large oil globules, although only one is apparent when the spore is viewed end on (Fig. 6, W). The wall is thickened and often bears a central projecting girdle of unknown composition (Fig. 6, X). The degradation of the host content is similar to that produced by the sporangium. In a number of specimens rhizoids have been observed which take the form of a few short threads (Fig. 6, V, W). Germination of the resting spore has not been seen. Two zygotes were seen bearing empty sporangia of a hyperparasite (Fig. 6, Z).

Although the method of formation of the resting spore is similar to that found in *Rhizophidium goniosporum*, *R. fallax*, and *R. planktonicum*, nevertheless the fungus here described cannot be identified with any of these species. In its sporangial characters it most closely resembles *R. transversum* (Braun) Rabenhorst. A few of Dangeard's (1900–1) drawings of this fungus are reproduced in Fig. 7, A–F. The most obvious difference is found in the asexual nature of the resting spore. Until more is known regarding this structure in *R. transversum* it is suggested that a new species should be erected, *R. ephippium*, taking its name from the saddle shape of the sporangium.

Rhizophidium ephippium sp. nov.

Thalli monocentrici, eucarpici, sporangiis ephippiiformis (8–15μ latis × 4–5μ altis), rhizoideis perpaucis muniti. *Sporangia* poris duobus lateralibus oppositis dehiscentes. *Zoosporae* globosae, 2μ diam., uniguttulatae, postice uniflagellatae. *Sporae* perdurantes ovatae vel subglobosae, 5–7 × 3·7–5μ, membrana levi hyalina, ex conjunctione cellulae masculae parvae cum cellula femina majore generatae (cellula mascula modo appendiculare persistenti), intus globulis duobus magnis refractis munitae.

Hab.: In cellulis vivis *Stylosphaeridii stipitati* Windermere, Esthwaite Water, et Blelham Tarn, Angliae.

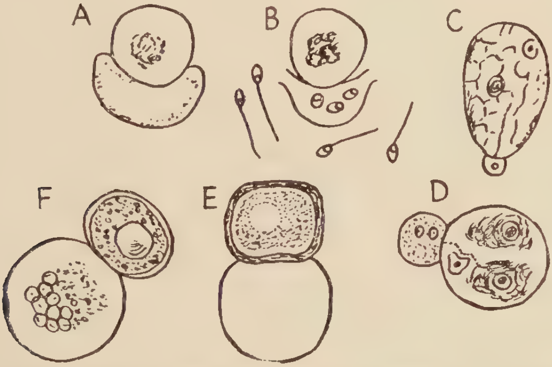

Fig. 7, A–F. *Rhizophidium transversum* (Braun) Rabenhorst. A–F, development and structure of *Chytridium transversum* A. Braun (after Dangeard 1900).

Rhizophidium ephippium n. sp.

Thallus eucarpic, epibiotic, sporangium saddle shaped, 8μ broad by 4μ high to 15μ broad by 5μ high, containing 8 to 30 zoospores. Zoospores spherical, 2μ in diameter, uniguttulate, posteriorly uniflagellate, emerging singly after dissolution of two oppositely directed lateral papillae. Intramatrical rhizoidal system composed of one or a few short threads. Resting spores oval to subspherical, 5μ broad by 3·7μ high to 7μ broad by 5μ high, arising from fusion of the content of a small male with a larger female thallus, the former remaining as an appendage to the mature resting spore. Wall smooth, colourless, beset with a narrow central projecting band of wall material, content consisting of two large refractive globules, germination unknown.

Parasitic on *Stylosphaeridium stipitatum* Geitler from Windermere North and South Basins and Esthwaite Water, in the English Lake District.

III. *Chytridium versatile* Scherffel

This fungus occurs together with *Rhizophidium Fragilariae* on *Fragilaria crotonensis*, but never in such large numbers as the latter. Neither *Asterionella formosa* nor *Melosira italica* has ever been observed to be parasitized by this chytrid. The obpiriform sporangium (11·9μ high by 7·4μ at its greatest diameter to 9·5μ high by 5·7μ diameter) tapers to a cup-shaped base resting on a short extramatrical stalk 1–2μ long. The intramatrical rhizoidal system was not observed. At maturity the sporangium contains 20–30 oil globules each indicating the position of a zoospore. Dehiscence of the sporangium is undoubtedly operculate; the convex operculum often remaining close to the empty sporangium (Fig. 8, E, F). The sporangium does not collapse after dehiscence. No resting spores have been found.

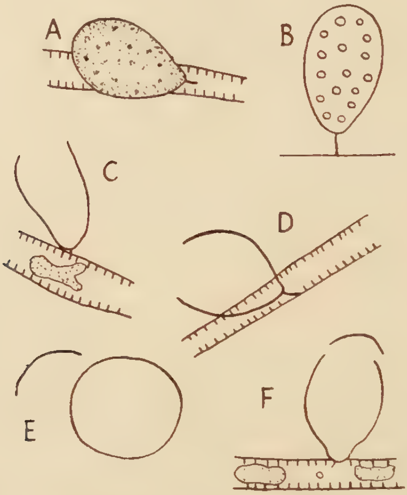

FIG. 8, A–F. *Chytridium versatile* Scherffel. A, immature sporangium. B, mature sporangium. C–F, empty sporangia, operculum present in E and F. (E) viewed from above. (A–F, × 1400.)

TABLE III

Occurrence in the Lake District

Lake.	Host.	1946.	1947.	1948.
Windermere: South Basin	*Fragilaria crotonensis* (A.M. Edw.) Kitton	Sept. 10–Oct. 23	Nov. 11–Dec. 22	Jan. 6 at intervals,–Oct. 26
	Tabellaria fenestrata (Lyngb) Kütz. var. *Asterionelloides.* Grün.	Oct. 8	Nov. 11	June 30–Aug. 3
Windermere: North Basin	*Fragilaria crotonensis*	Oct. 20, Nov. 20	—	July 26–Aug. 3
	Tabellaria fenestrata var. *Asterionelloides*	Nov.	—	—
Esthwaite Water	*Fragilaria crotonensis*	Oct. 28	—	—

IV. *RHIZOPHIDIUM FRAGILARIAE* N. SP.

TABLE IV

Occurrence of R. Fragilariae *on* Fragilaria crotonensis (*A. M. Edw.*) *Kitton*

Lake.	1946.	1947.	1948.
Windermere: South Basin	Sept. 2–Oct. 30, Dec. 12	Feb. 5–June 18, July 4, Nov. 11–Dec. 22	Jan. 6–Nov. 10
Windermere: North Basin	—	Oct. 20–Dec. 1	Apr. 20–Nov. 22
Esthwaite Water	Apr. 18, Aug. 27–Nov. 14	—	Apr. 2–Nov. 16

This parasite (Fig. 9, A–G) appears to be limited to *Fragilaria crotonensis*, as it has never been observed on any other diatom of the plankton. The sessile sporangia are spherical to subspherical and vary greatly in size; small ones, 3μ in diameter, produce about three zoospores, while the largest, 10μ in diameter, may contain as many as twenty zoospores. The majority range from 5·2 to 7·7μ in diameter. The intramatrical rhizoidal system, where visible, consists of a short unbranched or once-branched thread. At maturity the sporangium wall deliquesces, forming one, two, or three pores through which the zoospores emerge (Fig. 9, G). At first they are entangled by their flagella, but after a few minutes they swim away individually. The zoospores are spherical, 2–2·4μ in diameter, with a conspicuous anterior oil globule and posterior flagellum. Although many thousands of specimens have been observed no resting spore has yet been found.

In spite of the fact that no distinctive characteristic can be cited for this chytrid it nevertheless seems so common and so definite in its host relations to warrant the erection of a new species. *Rhizophidium Fragilariae* is suggested.

Rhizophidium Fragilariae sp. nov.

Thallus e sporangio externo et parte rhizoidea interna brevi haud vel singulariter ramosa compositus. *Sporangium* globosum vel subglobosum, 3–10μ diam., 3–20 zoosporas includens; tunica in maturitate deliquescenti, et apertiones singulas ad tres, per quas zoosporae abeunt, efformante. *Zoosporae* globosae, 2–2·4μ, globulo conspicuo anteriore et flagello singulo simplice posteriore praeditae.

Sporae perdurantes nondum visae.

Hab.: In *Fragilaria crotonensi* (A.M. Edw.) Kitton viventi parasitica in lacubus Esthwaite Water et Windermere, prope Wray Castle, Ambleside, Angliae.

Rhizophidium Fragilariae n. sp.

Sporangia epibiotic, spherical to subspherical, 3–10μ in diameter, containing 3 to 20 zoospores. Sporangium wall deliquesces at maturity, forming 1 to 3 openings through which the zoospores emerge. Zoospore spherical, 2–2·4μ, with a conspicuous anterior globule and single posterior flagellum. Endobiotic rhizoid a short unbranched or once-branched thread. Resting spore not observed.

Parasitic on *Fragilaria crotonensis* (A. M. Edw.) Kitton in Esthwaite Water and Windermere, near Wray Castle, Ambleside, England.

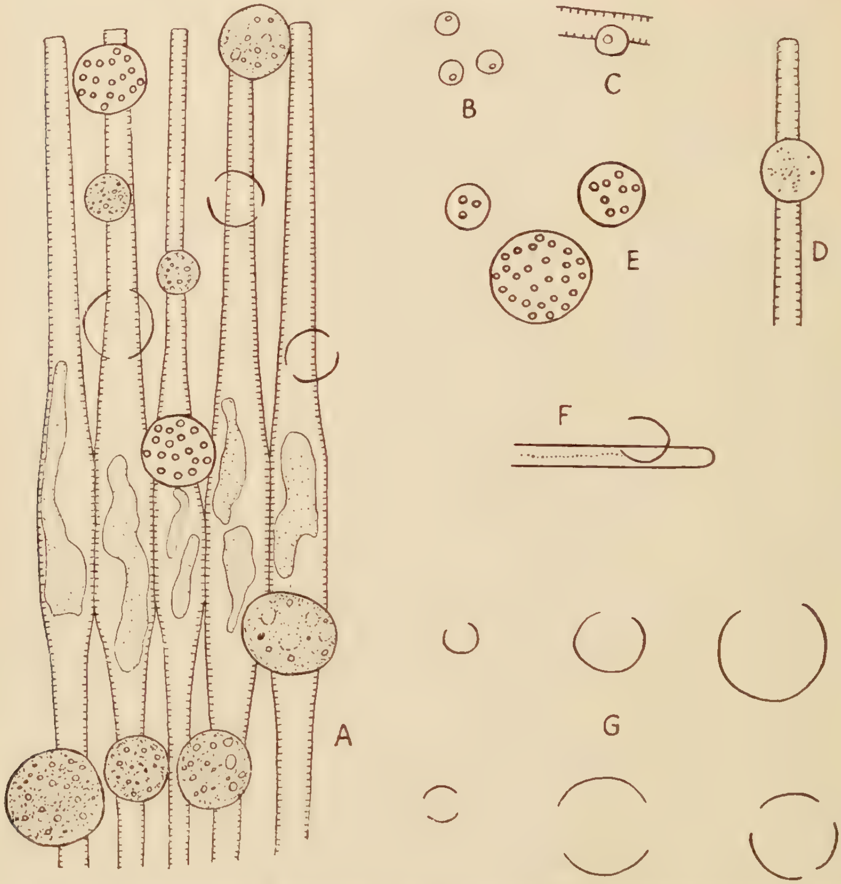

FIG. 9, A–G. *Rhizophidium Fragilariae* n. sp. A, portion of a *Fragilaria* filament bearing young, mature, and empty sporangia. B, zoospores. C, recently encysted zoospore. D, immature sporangium. E, mature sporangia. F, empty sporangium and its rhizoid. G, empty sporangia showing variation in size and number of the dehiscence pores. (A–E, G, × 1400; F, × 1066.)

V. *RHIZOPHIDIUM SPHAEROCYSTIDIS* N. SP.

TABLE V

Occurrence on Sphaerocystis Schroeteri *Chod.*

Lake.	1947.
Windermere: North Basin .	July 8–25
Windermere: South Basin .	July 1–15
Blelham Tarn . . .	July 11–Aug. 19
Esthwaite Water . .	July 4–10

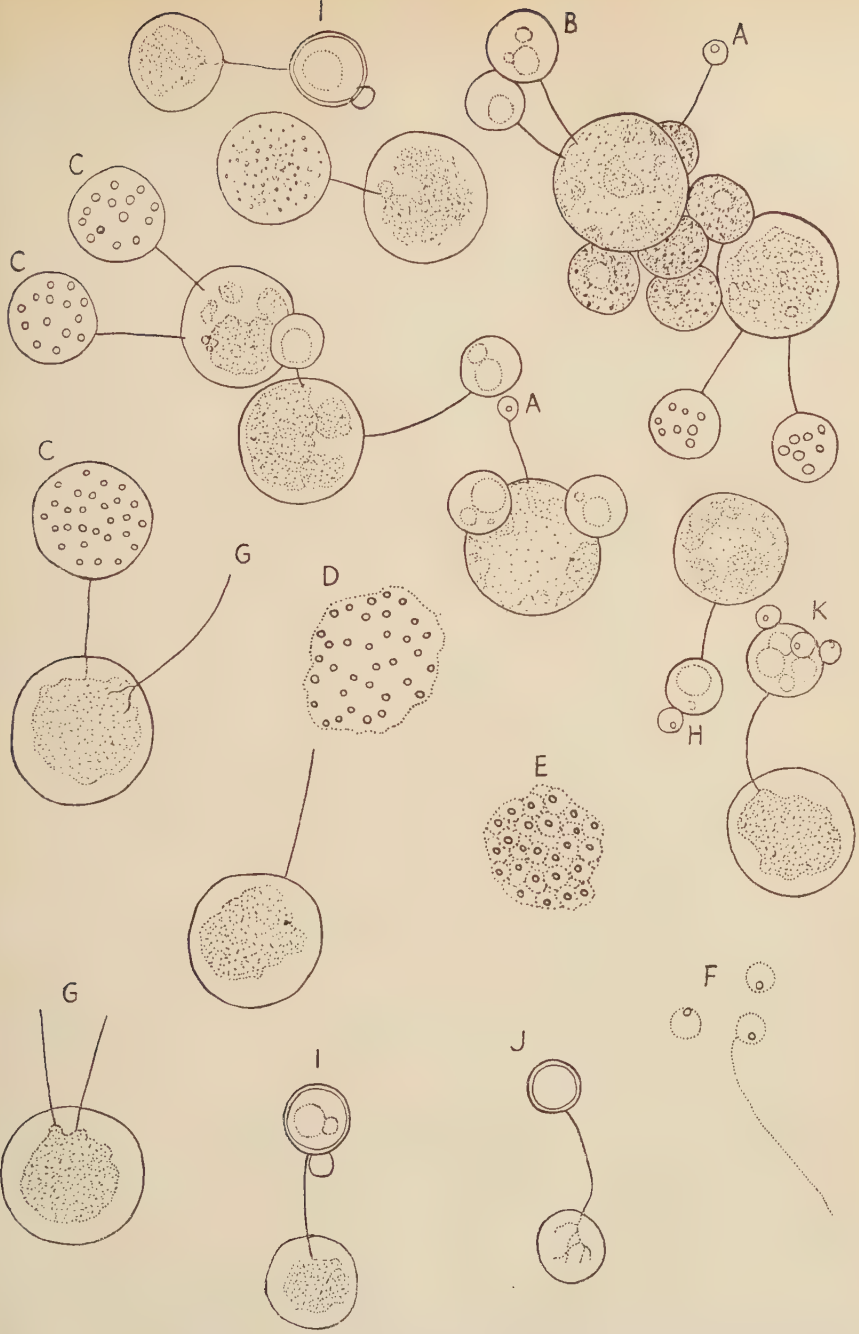

FIG. 10, A–K. *Rhizophidium Sphaerocystidis* n. sp. A, recently encysted zoospores with a fine germ-tube. B, young sporangia. C, mature sporangia. D, dehiscing sporangium. E, zoospore mass. F, zoospores. G, stalk-like portion which remains after dehiscence of a sporangium. H, immature resting spore. I, mature resting spore with empty adherent male cell. J, resting spore mounted in lactophenol and cotton blue to show its rhizoidal system. K, for explanation see text page 279. (All × 1400.)

The encysted zoospore on the mucilage sheath surrounding its host develops a straight rhizoid which penetrates the mucilage, enters the algal cell, and forms inside a meagre branched rhizoidal system, often difficult or impossible to distinguish owing to the dense content of the host. The encysted zoospore enlarges to form a thin-walled spherical sporangium and the germ-tube remains very narrow like a stalk. The mature sporangia (Fig. 10, C) vary from 5 to 11 μ in diameter and contain from 8 to 50 zoospores. On dehiscence the sporangium wall gradually disappears, leaving a protoplasmic mass which becomes irregular (Fig. 10, D) and ultimately cleaves into the zoospores (Fig. 10, E, F). The rhizoid alone indicates the position of a former sporangium (Fig. 10, G). The zoospore is spherical (3 μ), uniguttulate with a long posterior flagellum. The resting spore is sexually formed and develops in a manner already described for *Rhizophidium goniosporum* Scherffel (1925) and *R. planktonicum* Canter (1948). The male gamete attaches itself directly to the wall of the larger female thallus already established on the host cell (Fig. 10, H). The mature zygote is spherical (5–7 μ), with a thick, smooth wall adherent to which is the empty male thallus (2·7 μ) (Fig. 10, I). The content consists of one or two large refractive globules. The presence of the chytridiaceous hyperparasite *Septosperma anomala* (Couch) Whiffen (1942), attacking all stages in

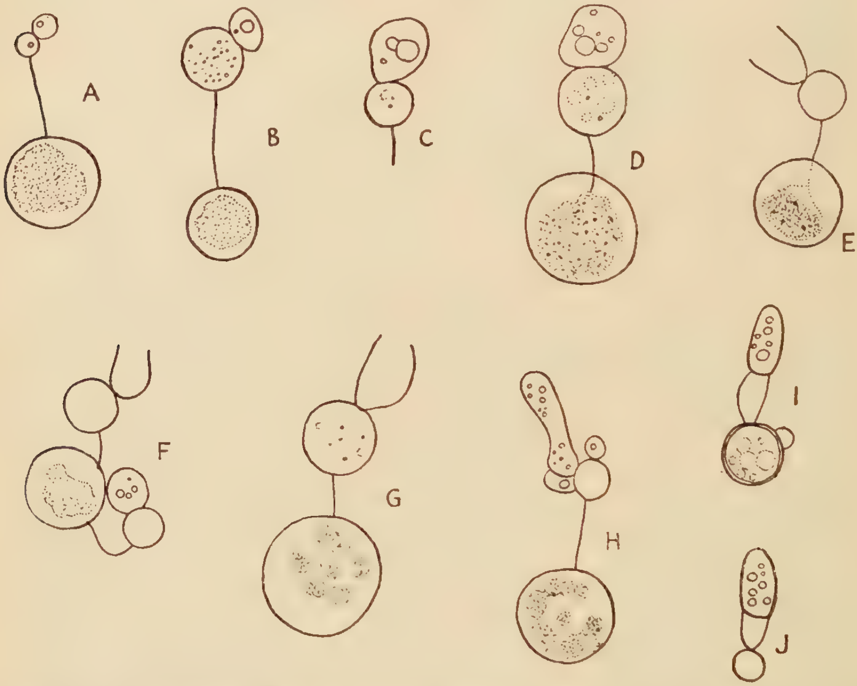

FIG. 11, A–J. *Septosperma anomalum* (Couch) Whiffen. A–D, stages in development of the sporangium. E–G, empty sporangia. H, young resting spore. I, J, mature resting spores. (A–C, F–J, × 1066; D, E, × 1400.)

the life-history of *Rhizophidium Sphaerocystidis*, adds difficulties to the interpretation of the sexual process. Fig. 11, A, can be interpreted in two ways. Either it is the earliest stage in sexual fusion, in which case this fungus would resemble *Phlyctidium Eudorinae* Gimensi (see Sparrow, 1943, p. 153) and *Loborhiza Metzneri* Hanson (1944) where the resting spore is formed after fusion of two isogamous gametes. Secondly, it may be an extreme instance in which a zoospore of *Septosperma* has settled on a zoospore of its host. The latter seems more likely as only one such specimen was observed. Again, it is possible that in Fig. 10, K, the adherent zoospore-like bodies are a mixture of hyperparasite zoospores and male gametes of *Rhizophidium Sphaerocystidis*.

R. Sphaerocystidis differs from the majority of the Chytridiales in the manner of dehiscence of the sporangium. So far three chytrids have been described in which discharge is by dissolution of the whole sporangium wall. They are placed in three monotypic genera, *Nowakowskia* Borzi, *Solutoparies* Whiffen (1942), and *Hapalopera* Fott. In *Nowakowskia* (cited from Sparrow, 1943, p. 288), after dissolution of the sporangium wall, the zoospore mass becomes divided into spore balls, which in their turn break up into smaller spheres. This behaviour is unlike any other chytrid, and I agree with Whiffen (1942) that any species placed in this genus should agree with the type species in this respect.

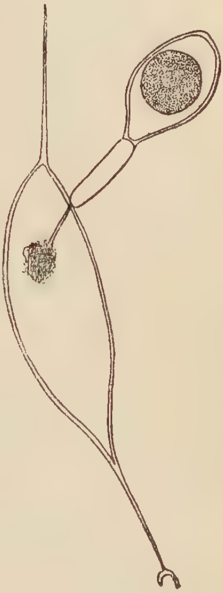

FIG. 12. *Hapalopera piriformis* Fott. Resting spore, from sole specimen observed by Dr. B. Fott in preserved material (drawing kindly sent to me by Dr. B. Fott).

The difference between *Hapalopera* and *Solutoparies*, however, are not so well marked. While the details of their morphology and their hosts differ, nevertheless they both exhibit a rhizidiaceous type of development. No resting spore is described for *Solutoparies*, and although in the diagnosis of *Hapalopera* Fott (1942) refers to a resting spore as spherical he does not figure or refer to it elsewhere in his paper. However, in a personal communication Dr. Fott kindly sent me a drawing of a resting spore he found in preserved material which I am reproducing with his permission (Fig. 12). No sexual process was observed.

In its method of sexual reproduction my fungus clearly resembles *Rhizophidium goniosporum* Scherffel and *R. planktonicum* Canter. Indeed, these new genera based on the method of dehiscence of the sporangium by deliquescence of the whole sporangium wall may well be unsatisfactory, for in *R. clinopus* Scherffel (1931) the entire upper half of the wall deliquesces, and De Wildeman (1931) sometimes noted total deliquescence of the wall in *R. Schroeteri*. It seems that the fungus here described is new to science, and in spite of its curious method of dehiscence it is suggested that for the time being it should be placed in the genus *Rhizophidium* with the binomial *R. Sphaerocystidis.*

Rhizophidium Sphaerocystidis sp. nov.

Thallus ex partibus tribus compositus; sporangio sessili in vagina mucosa hospitali externe disposito, parte stipitali tereti per vaginam mucosam penetrante, et parte rhizoidea intra cellulam hospitalem ramosa. *Sporangium* globosum (5–11 μ) zoosporas 8–50 includens, tunica in maturitate perfecte deliquescente circumdatum. *Zoosporae* globosae (3 μ), uniguttulatae, ex posteriore uniflagellatae, quandocumque perfecte maturescunt ex sporangio natante. *Sporae perdurantes* globosae (5–7 μ), tunica levi praeditae, globulos oleaceos magnos singulos duosve includentes; per conjugium cellulae masculinae parvae cum cellula femina majore ortae, illa ut appendicula persistente. *Germinatio* nondum visa.

Hab.: In *Sphaerocystide schroeteri*, in lacubus Windermere, Esthwaite Water, et Blelham Tarn, prope Wray Castle, Ambleside, Angliae.

Rhizophidium Sphaerocystidis n. sp.

Thallus consisting of a sporangium sessile on the outside of the mucilage sheath surrounding the host, an unswollen stalk-like region within the mucilage, and a branched rhizoidal system inside the host cell.

Sporangium spherical (5–11 μ) containing 8 to 50 zoospores. At maturity the entire sporangium wall deliquescing and the zoospores when fully delimited swim away. Zoospores spherical (3 μ), uniguttulate, posteriorly uniflagellate.

Resting spores spherical (5–7 μ), wall smooth, containing one or two large oil globules; formed after fusion of a small male cell with a larger female, the former remaining as an appendage to the mature spore. Germination unknown.

Parasitic on *Sphaerocystis Schroeteri* Chod. in Windermere, Esthwaite Water, and Blelham Tarn, near Wray Castle, Ambleside, England.

VI. *ZYGORHIZIDIUM MELOSIRAE* N. SP.

TABLE VI

Occurrence of Z. Melosirae *on* Melosira italica (*Ehrenb.*) *Kütz.*

Lake.	1945.	1946.	1947.	1948.	1949.
Esthwaite Water	Oct. 25	Jan. 5–Apr. 5 Oct. 28, Dec. 30	Jan. 1–Apr. 4 Oct. 16–Dec. 29	Jan. 1–Apr. 12 Oct. 27, Dec. 14	Feb. 15–Apr. 27
Blelham Tarn	Nov. 3–Dec. 24	Oct. 7–Dec. 30	Jan. 6–Apr. 8 Nov. 5–Dec. 30	Jan. 7–Apr. 22 Oct. 28–Dec. 30	Jan. 12–May 9
Windermere: South Basin	—	Feb. 6–Mar. 6	Jan. 8–Apr. 17	Jan. 6–Mar. 16	Mar. 23–Apr. 20
Windermere: North Basin	—	—	—	Jan. 5, Feb. 9, Dec. 13	Jan. 24–May 9
Hawes Water	—	—	—	—	Feb. 3–Apr. 7
Ullswater, West	—	—	—	—	Mar. 3–Apr. 13
Ullswater, East	—	—	—	—	Feb. 3–Apr. 13
Loughrigg Tarn	—	—	—	—	Mar. 21

Both resting and dividing cells of *Melosira* are attacked and the contents reduced to a few small chocolate-brown spheres. The extramatrical sessile or stalked sporangia are ovate and vary from 7 to 14μ high by 5 to 10μ in diameter. The first rhizoid of the encysted zoospore either penetrates the diatom cell immediately or grows for a short distance outside, either free from or running along the surface of the host wall. In examples of the latter the sporangium is often located on a healthy cell, whereas a neighbouring one into which the rhizoids have penetrated is dead (Fig. 13, N).

The Hawes Water form of *Melosira italica* is surrounded by a wide mucilage sheath upon the outside of which the zoospore encysts (Fig. 13, B). A short tube penetrates the mucilage and remains as a stalk to the sporangium.

The intramatrical rhizoidal system is difficult to observe and sometimes cannot be seen, but where visible it consists of a short unbranched or branched thread which does not appear to taper (Fig. 13, N). At maturity the sporangium contains from 6 to 30 relatively large oil globules, each marking the position of a zoospore (Fig. 13, H, I). On dehiscence the unthickened apex of the sporangium separates as a lid. The first few zoospores emerge together and remain a few seconds at the orifice before swimming away; the rest are liberated singly. The zoospore is spherical, 3·3–3·8μ in diameter, with a large oil globule (0·9μ) and a single posterior flagellum. The empty sporangium does not collapse on dehiscence (Fig. 13, J–L).

The extramatrical resting spore, 9·5–10μ high by 6·1–7·5μ in diameter, is similar in shape to the sporangium but has a thick, smooth, highly refractive wall and at maturity contains a few large oil globules (Fig. 13, Q, R). It is formed after fusion of the contents of a small male cell (essentially an encysted zoospore) with those of a larger female thallus. The spherical male cell, 3–3·3μ in diameter, is connected to the lateral wall of the female by a conjugation tube, 0·7–7·5μ long by 1·0μ broad (Fig. 13, O–R). The rhizoidal system of the female thallus is like that of the sporangium, but no rhizoids have been seen attached to the male. Where the male is situated on a different host cell from the female, the host content of this cell remains quite healthy. The earliest observed stage in the union between the two thalli is shown in Fig. 13, O. The method of germination of the resting spore is unknown.

This chytrid, by the possession of an operculum and an epibiotic, sexually formed resting spore clearly belongs to the genus *Zygorhizidium*. It differs, however, from the type species *Z. Willei* Löwenthal (1905) in the shape of the sporangium and resting spore and in the absence of a subsporangial swelling, although in the type species the latter is not a constant feature. Scherffel (1925) noted that the non-sexual thalli of the form on *Mougeotia* differed from those on *Cylindrocystis* in the possession of a group of short, rod-like rhizoids, instead of an endobiotic knob. Only one other species has been described, namely *Z. verrucosum* Geitler (1942), in which the wall of the resting spore is covered with wart-like processes. The species here described clearly does not belong to either of the already known ones, and the binomial *Z. melosirae* is suggested for it.

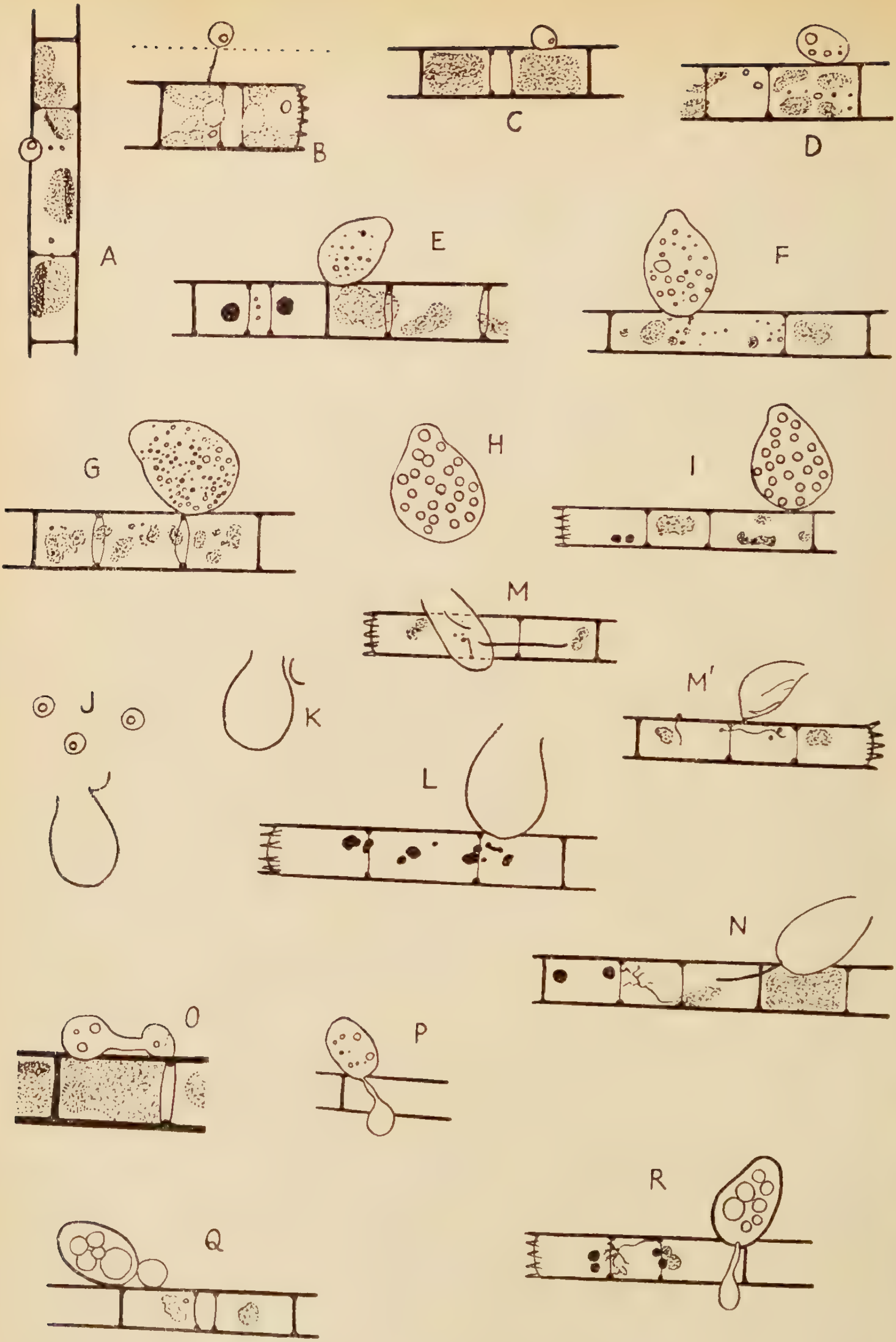

FIG. 13, A–R. *Zygorhizidium Melosirae* n. sp. A, encysted zoospore. B, the same as A but *Melosira* filament is surrounded by a mucilage sheath, which is indicated by a dotted line. C–G, stages in growth of the sporangium. H, I, mature sporangia. J, K, empty sporangia with opercula; zoospores. L–N, empty sporangia. M, M$^{\mathrm{I}}$, the same sporangia in different views; in M the portion of the rhizoid outside the diatom cell is visible, in M$^{\mathrm{I}}$ the endobiotic portion. O, young male and female thallus united by a conjugation tube. P, immature resting spore. Q, R, mature resting spores. (B, × 1066; O, × 1400; Rest, × 800.)

Zygorhizidium Melosirae sp. nov.

Thallus epibioticus, sessilis vel pedicellatus. *Sporangium* ovatum, 7–14μ altum, 5–10μ latum, zoosporas 8–30 continente, operculo dehiscente. *Zoosporae* sphaericae, 2·8–3·3μ diam., guttula oleosa magna (0·9μ), postice uniflagellatae. *Rhizoideum* intramatricale, simplex vel sparsim ramosum, aequale. *Sporae perdurantes* ovatae 9·5–10μ altae, 6–7·5 latae, laeves, intus paucis globulis praeditae, per conjugium cellularum sexualium ortae, cellula mascula parva cum cellula feminea majore per tubulum 0·7–1·5μ longum conjugante. *Germinatio* nondum visa.

Hab: In *Melosiram italicam* parasiticus, Esthwaite Water, Blelham Tarn, North Basin et South Basin, Windermere, Ullswater, Hawes Water et Loughrigg Tarn, Anglia.

Zygorhizidium Melosirae n. sp.

Thallus epibiotic, stalked or sessile, sporangium ovate 7–14μ high by 5–10μ in diameter with 6 to 30 zoospores, dehiscing by a lid. Zoospores spherical, 2·8–3·3μ in diameter, with a large oil globule (0·9μ), and posterior flagellum. Intramatrical rhizoid unbranched or sparingly branched, not tapering. Resting spores ovate, 9·5–10μ high by 6–7·5μ broad, wall smooth, the content with a few large globules; arising from fusion of the contents of a small male with a larger female cell by a conjugation tube, 0·7–7·5μ long. Germination unknown.

Parasitic on *Melosira italica* in Esthwaite Water, Blelham Tarn, Windermere North and South Basins, Ullswater, Hawes Water and Loughrigg Tarn, the English Lake District.

VII. *ZYGORHIZIDIUM PARVUM* N. SP.

TABLE VII

Occurrence of Z. parvum *in the English Lake District*

Lake.	Host.	1947.	1948.	1949.
Blelham Tarn	*Sphaerocystis Schroeteri* Chod.	June 19–Sept. 10	May 14–July 22	—
	Kirchneriella obesa (W. West) Schmidle	July 11	—	—
Esthwaite Water	*Sphaerocystis Schroeteri*	June 26–Aug. 28, Oct. 16	Apr. 20–July 28	—
Windermere: South Basin	*Sphaerocystis Schroeteri*	July 1–15	June 16–July 14	Apr. 6–May 10
Windermere: North Basin	*Sphaerocystis Schroeteri*	July 8–25	June 1–July 12	Apr. 19–May 9

Sphaerocystis Schroeteri and *Kirchneriella obesa* are both surrounded by a wide mucilage sheath. The sporangium develops from the encysted zoospore and a part or the whole of the germ-tube (Fig. 14, A, B). When mature it varies

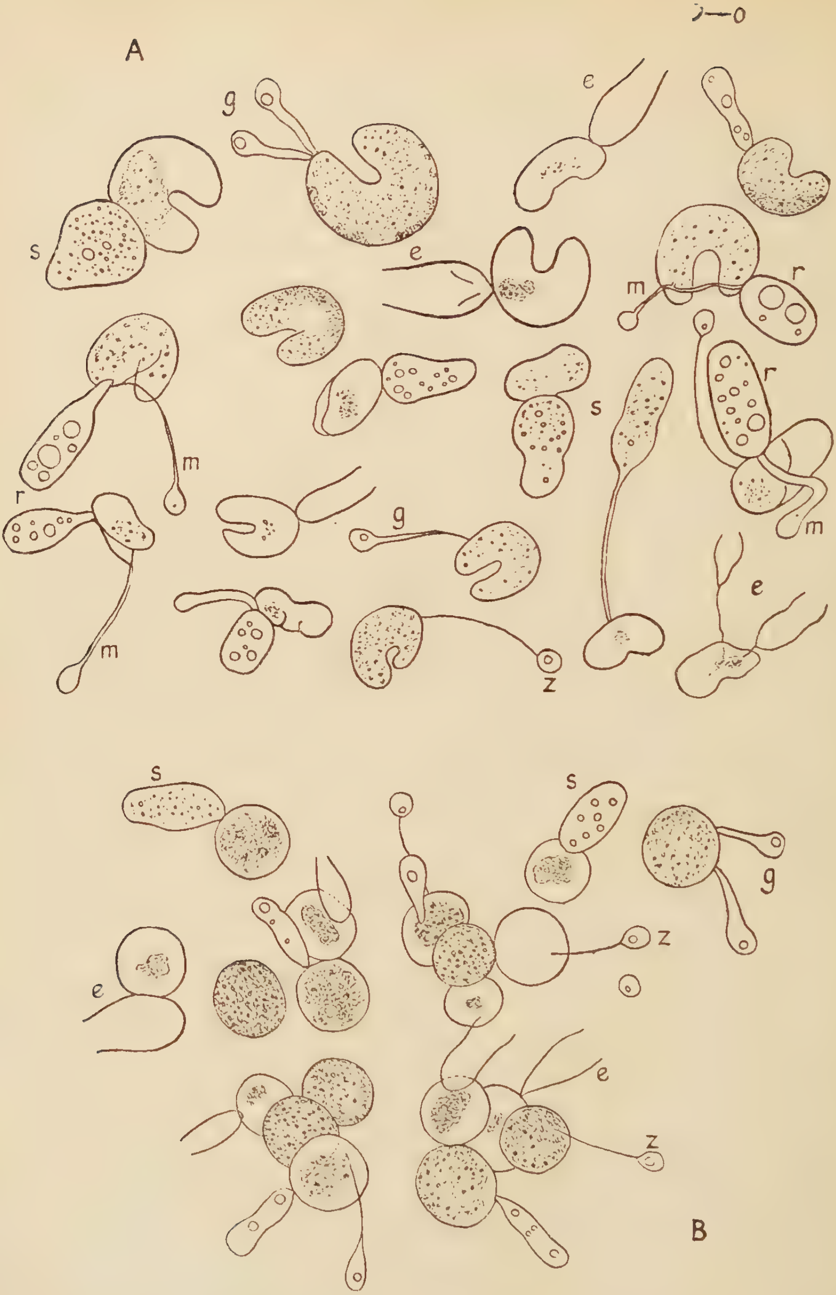

FIG. 14, A, B. *Zygorhizidium parvum* n. sp. A, *Kirchneriella*, B, *Sphaerocystis* cells bearing the parasite in various stages of development. *e*, empty sporangia; *g*, broadening of germ-tube; *m*, male thallus; *o*, operculum; *r*, resting spore; *s*, sporangia; *z*, encysted zoospore with germ-tube. (× 1400.)

in shape from piriform, measuring 4·7μ high by 2·3μ broad to 13μ high by 8μ broad, to cylindrical, 9·5μ high by 1·9μ broad. The sporangium may be sessile on the host cell or stalked. If there is a stalk it is simply part of the original germ-tube which has not swollen. Both mother and daughter algal cells are parasitized, the mother cells bearing the larger sporangia. There are from 4 to 40 zoospores in a sporangium, and when it is narrow and elongate the zoospores are arranged in a linear series. The zoospores are fully formed within the sporangium, and several emerge together when the lid becomes detached; the rest follow in rapid succession. The zoospores (1·9μ in diameter) are spherical, with a relatively large anterio-lateral globule and accompanied by a much smaller one.

The rhizoidal system appears to be of limited extent and where visible consists of a small unbranched thread.

Resting spores formed by a sexual process are produced in quantity. Their method of formation is similar to that described for *Zygorhizidium* spp. (see Löwenthal, 1905; Geitler, 1942) and *Rhizophidium columnaris* Canter (1947). Contact is established between a minute male thallus (essentially an encysted zoospore and its germ-tube) with a slightly larger female thallus by means of a conjugation tube which arises as a lateral branch from the male, passing through mucilage until it reaches a female (Fig. 15, H–L). Although it is presumed that the germ-tube of the male enters the host cell to which it is attached, no internal rhizoidal system has been observed and such host cells show little sign of disease. The content of the male passes into the female, which then enlarges into the resting spore. The fusing gametes are very slightly different in size (Fig. 15, H, I). At first the resting spore contains a few large oil globules, but when mature there are more numerous smaller globules. The resting spore may be spherical (6–8μ) or elongate oval (5·3μ high by 3·5μ broad to 15μ high by 8μ broad); it is always surrounded by a thick, colourless, smooth wall (Fig. 15, L).

As has been recorded for *Zygorhizoidium Willei* Löwenthal (1905) and *Rhizophidium columnaris* Canter (1947), male thalli which have found no female with which to fuse may then grow into sporangia (Fig. 15, M).

One other parasite has been recorded on *Sphaerocystis Schroeteri* from the plankton of the Walensee by Huber-Pestalozzi (1946, p. 99, fig. 6); see Fig. 16. Huber-Pestalozzi, however, neither identified it with a known species nor described it under a new binomial. It may be identical with the British form since the sporangia are of similar shape and develop in the same manner. However, no resting spores were observed, nor is it known whether the sporangium is operculate.

Once again difficulties are presented in relation to the generic position of this chytrid. Although the method of formation of the resting spore is similar to the operculate *Zygorhizidium*, nevertheless it differs in the fact that both the sporangium and resting spore develop from the encysted zoospore and its germ-tube. The fungus under consideration is parallel with *Dangeardia mammillata* (Canter, 1946) among the inoperculate chytrids.

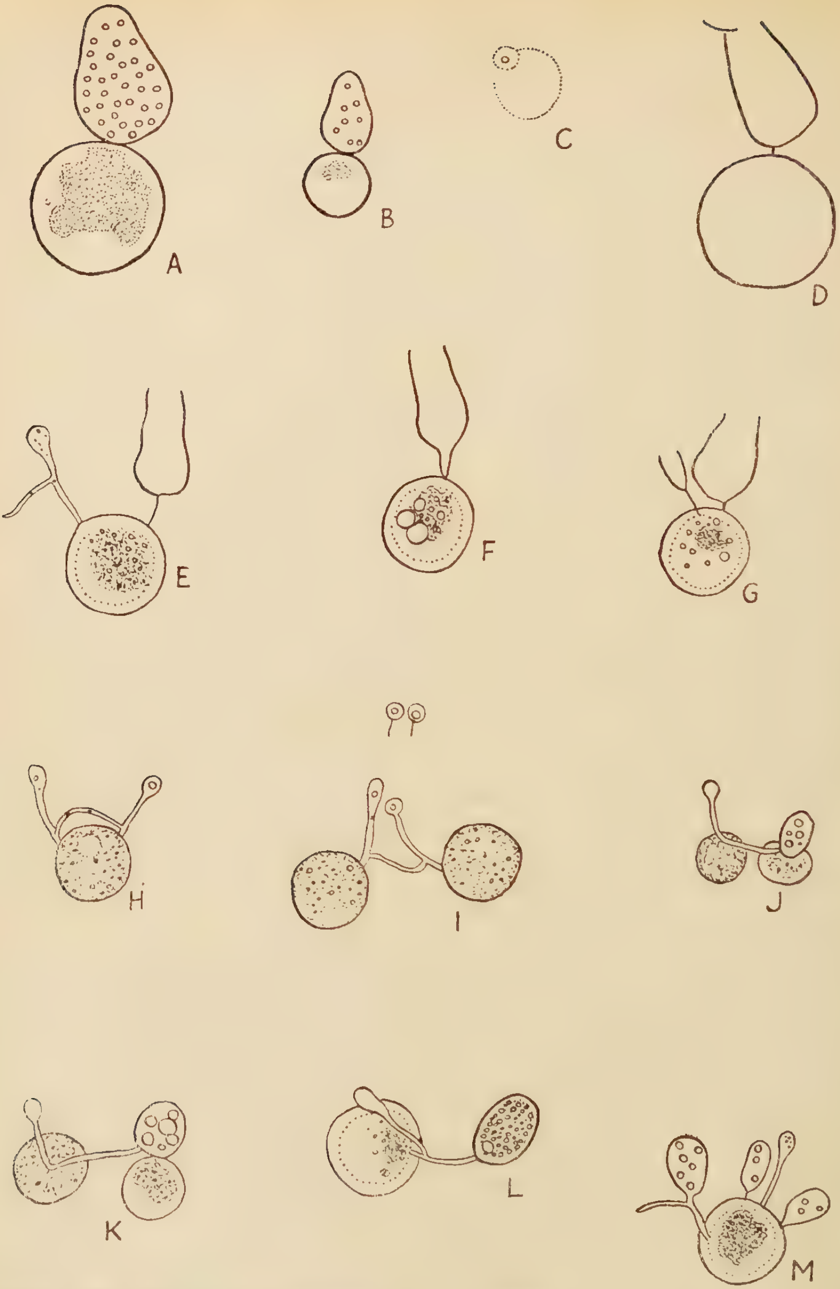

FIG. 15, A–M. *Zygorhizidium parvum* n. sp. A, B, mature sporangia. C, zoospore. D–G, empty sporangia. E, empty sporangium and male thallus which shows signs of developing into a sporangium. H, I, conjugating thalli; a slight anisogamy of the gametes is noticeable. J, K, immature resting spores. L, mature resting spore. M, host cell bearing small sporangia, and a male thallus which has developed into a zoosporangium. (A–D × 1400; E–M × 1130.)

Although it may be that a new genus of the operculate series should be erected for this fungus, in our present incomplete knowledge of these forms and characters on which to base generic distinction it seems best to include it in the genus *Zygorhizidium* as a new species *Z. parvum.*

Zygorhizidium parvum sp. nov.

Thalli monocentrici, eucarpici, sporangiis et rhizoideis brevibus tenuibus simplicibus muniti. *Sporangia* piriformia, cylindrica vel ovata, 5–13 × 2·5–8μ, per operculam dehiscentes, zoosporis 4–40, postice uniflagellatis, 2μ diam. *Sporae perdurantes* globosae, 6–8μ diam., vel ovatae 5·3–15 × 3·5–8μ, membrana levi, multiguttulatae, sexualiter formatae (ut in aliis speciebus generis) sed thalli masculi tubo conjugationis laterali e tubo germinationis emergenti.

Hab.: In cellulis vivis *Sphaerocystidis Schroeterii*, Blelham Tarn, Esthwaite Water et Windermere, et in cellulis vivis *Kirchneriellae obesae*, Blelham Tarn, prope Wray Castle, Angliae.

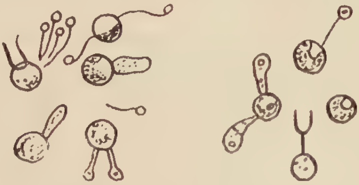

FIG. 16. Parasite on *Sphaerocystis Schroeteri* Chod. from the Walensee (after Huber-Pestalozzi 1946 Abb. 6).

Zygorhizidium parvum n. sp.

Thallus monocentric, eucarpic, consisting of a sporangium developed from the body of an encysted zoospore and the whole or a part of the original germ-tube. Sporangia pear-shaped, cylindrical, or oval, varying in size from 5 to 13μ high by 2·5 to 8μ broad. Zoospores from 4 to 40 fully formed within the sporangium, emerging after the detachment of a lid. Zoospores, 2μ in diameter, posteriorly uniflagellate, with an anterior lateral globule and a minute shining granule. Rhizoid a short, unbranched thread. Resting spore sexually formed male thallus (resembling an encysted zoospore and its germ-tube) makes contact with a slightly larger female thallus by means of a conjugation tube which arises as a lateral branch from the germ-tube of the male. Resting spore spherical, 6–8μ in diameter, or elongate-oval, 5–15μ × 3·5–8μ. Wall smooth, hyaline, content composed of small globules. Germination unknown.

Parasitic on *Sphaerocystis Schroeteri* Chod. in Blelham Tarn, Esthwaite Water, and Windermere, and on *Kirchneriella obesa* (W. West) Schmidle, in Blelham Tarn, near Wray Castle, the Lake District, England.

RECENT RECORDS OF PHYTOPLANKTON PARASITES FROM OTHER COUNTRIES

The fungal parasites of planktonic algae have received little attention from students of chytrids, and particulars of their distribution are few. In recent years two workers who are primarily algologists have described parasites from Switzerland and Sweden. Huber-Pestalozzi (1946) figures chytrids on *Asterionella formosa* Hass and *Sphaerocystis Schroeteri* Chod. from the Walensee and on *Fragilaria capucina* from Zürichsee. Earlier this worker (1944) describes *Chytridium oocystidis* n. sp. parasitizing *Oocystis lacustris* Chod. in the Walensee.

The Swedish records are by Skuja (1948, pp. 379–82). *Olpidium entophytum* (A. Braun) Rabenhorst is described in *Gloeocystis bacillus* and *Gloeocystis planktonica* from Erken.

A very similar parasite occurs in *Gloeocystis* in the English Lake District. All the details of its life-history are not known, but it produces biflagellate zoospores. Skuja never observed the zoospores in his form and it is possible, especially from his drawing (1948, Taf. XXXIX, Fig. 7), that this species is also a biflagellate and does not belong in the genus *Olpidium*. *Olpidium endogenum* (Braun) Schroeter is described from Arnsjou in *Cosmarium depressum* var. *achondrum*. Skuja does not refer to the zoospores of this fungus and again it may be biflagellate, in which case it could be referred to a dwarf form of *Myzocytium megastomum* De Wildeman.

Phlyctidium anabaena Rödhe and Skuja is a new species parasitic in young resting spores of *Anabaena* spp. from Säbysjön. *Chytridium microcystidis* Skuja parasitizes *Microcystis* spp. in Erken.

The remaining parasites occur on *Melosira ambigua*. They are *Rhizophidium simplex* (Dang.) Fischer from Erken, *Rhizophidium fusus* (Zopf) Fischer from Örsjön, and *Chytridium versatile* Scherffel from Örsjön, Bredsjön, and Ubby-Långsjön.

It is unlikely that the lakes in the English Lake District are exceptional in the large variety of algal parasites they contain. It is probable that if other lakes with a comparable algal flora were constantly studied, they too would be found to possess a similar parasitic fungal flora.

Encouraging results have been obtained from observations on single plankton samples[1] (preserved in formalin) from twenty-five lakes in Switzerland. In ten of these samples parasites were present.

In the Pfaffikersee one specimen of a polyphagous chytrid was found on *Gemellicystis* and on *Sphaerocystis*, a fungus identical with one figured by Huber-Pestalozzi (Fig. 16) from the Walensee. It is possible that these fungi are *Rhizidium windermerense* (p. 265) and *Zygorhizidium parvum* (p. 283) respectively. Again, in this sample *Eudorina elegans* was heavily parasitized by a fungus resembling *Endocoenobium Eudorinae* Ingold (1940).

Although the value of preserved samples is limited for obtaining a sound knowledge of any one fungus life-history, these nevertheless do indicate that in Switzerland there is a wide field open to the enthusiastic aquatic mycologist.

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[1] Kindly collected for the International Limnological Congress in Zürich, 1948, under the supervision of Professor O. Jaag.

SUMMARY

The phytoplankton of four bodies of water in the English Lake District have been examined from October 1946 to April 1949 for the presence of aquatic fungi. A survey of seventeen other lakes was begun in January 1949. Forty different fungi have been observed, seven of which are described in this paper, namely, *Rhizophidium ephippium* n. sp., *R. Fragilariae* n. sp., *R. Sphaerocystidis* n. sp., *Rhizidium windermerense* n. sp., *Chytridium versatile* Scherffel, *Zygorhizidium Melosirae* n. sp., and *Z. parvum* n. sp.

My thanks are due to Mr. D. Gawen for collecting the samples from Windermere, Esthwaite Water, and Blelham Tarn, and especially to Professor C. T. Ingold for his criticism of the manuscript and for all the encouragement he has given throughout the course of this work. For the Latin diagnoses my thanks are due to Miss E. M. Wakefield of Kew, and to Mrs. F. Balfour-Brown of the British Museum.

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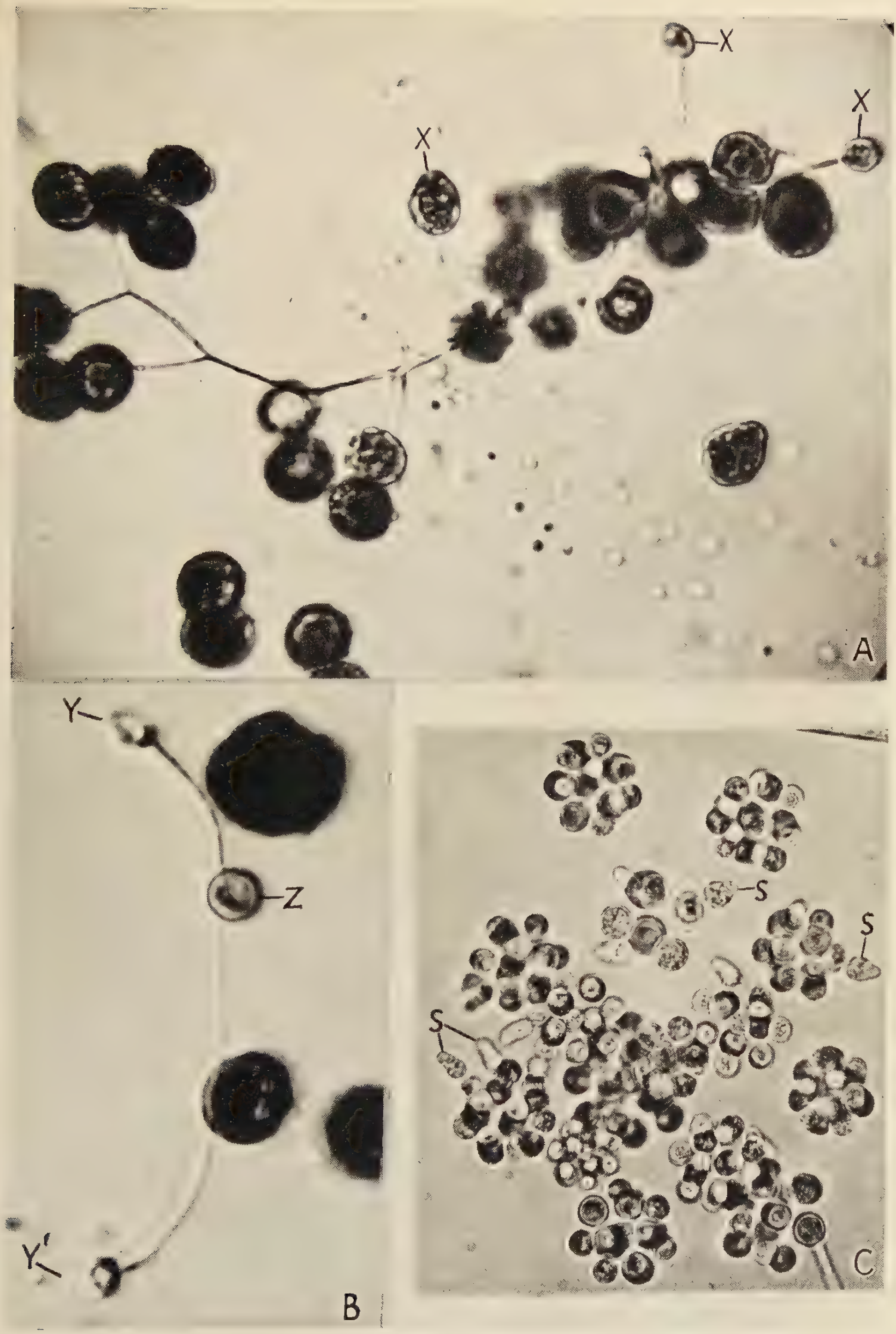

A. Part of a colony of *Gemellicystis neglecta* bearing three sporangia (*X*) of *Rhizidium windermerensis*. n. sp. The extensive rhizoidal system is clearly visible × 1764.

B, *Rhizidium windermerensis* n. sp. Two conjugating thalli (*Y*, Y^1). The spherical body (*Z*) is the incipient resting spore or zygote produced at the point of fusion of the conjugation tubes × 2200.

C. Several pear-shaped sporangia (*S*) of *Zygorhizidium parvum* n. sp. on *Sphaerocystis Schroeteri* × 600.

H. M. CANTER.

Growth Analysis of Cacao Seedlings

BY

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With six Figures in the Text

THE programme of physiological investigations on cacao (*Theobroma cacao*) planned at the West African Cacao Research Institute includes a detailed study of the effects of external factors, particularly light, on seedling growth. With this in view, it was necessary to study the variability of the various changes by which growth can be expressed, to develop methods by which the precision of estimates of these changes could be improved, and to determine the degree of replication which would be required. Hence a series of preliminary observations was conducted under ordinary field conditions, without differential treatments, which would give information as to the normal growth characteristics of cacao and at the same time enable the questions mentioned to be answered.

The present study is an account of these preliminary observations, which extended over a period of 7 months from planting; it appears to be the first such investigation performed on a tree crop. An exhaustive study, on similar lines, of the first 6 weeks of development of the cacao seedling has already been published (Goodall, 1949), and hence the present paper will concern itself mainly with the period between 6 weeks (when the first 'flush' of leaves has almost completed expansion and the cotyledons are nearly ready to fall) and 7 months from planting. It should be explained that the leaves of cacao do not expand in regular succession, but that a group of leaves formed by the same apex expand almost simultaneously, and that an interval of several weeks then elapses before the expansion of the next 'flush'. During the period covered by these observations a total of either five or six 'flushes' expanded on each plant.

MATERIAL AND METHODS

Planting material consisted of seeds taken from two pods of each of four West African Amelonado trees. The testas were removed, and 55 seeds taken at random from the progeny of each parent were weighed. The 220 seeds were planted on August 16, 1946, in individual baskets in a nursery under *Leucaena glauca*. This leguminous shade tree, native to America, is commonly used in nurseries and young plantations of cacao, and the light intensity under it was some 20 per cent. of full daylight.

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[1] Now at Botany School, University of Melbourne, Australia.

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Seeds from each parent were taken at the time of planting for dry-weight determinations. The mean loss in weight on drying was 25·8±0·9 per cent., and did not vary with parentage.

The baskets were inspected daily and the date of emergence from the soil noted in each case, as well as that on which the cotyledons separated and the plumule became visible. Shortly after this daily measurements of the stem height (from the cotyledons) and of the length and maximum breadth of each growing leaf commenced.

Plants were harvested at 6, 12, 18, 24, and 30 weeks from planting. On each of the first four occasions 10 plants of each progency were selected, but for the last harvest only 5 plants of each progeny were available. Apart from a small number of failures in germination, a considerable amount of insect damage occurred after 3 months, and the more severely damaged plants were rejected.

At harvest the plants were brought in their baskets to the laboratory and the stem of each was severed immediately below the cotyledonary node. The roots were carefully washed. The leaves were separated and cleaned with a brush. Each leaf, stem, and root, and the cotyledons while they were present, were weighed separately, dried in a steam-oven for at least 24 hours, and re-weighed. Between weighing and placing in the oven a print or outline was made of each leaf for subsequent area determination by the methods already described (Goodall, 1947, 1949).

In addition to these records an attempt was made to collect any parts lost by the plants during development—the cotyledons, when they abscissed at about 8 weeks; young leaves which frequently also fall by abscission before reaching maturity; and leaves lost through insect damage. It was unfortunately impracticable to estimate, day by day, the loss of leaf material through leaf-eating insects removing a part of the lamina.

The principal purpose of the work being to estimate, as closely as possible, the changes in dry weight and amount of water in the plant during successive 6-week periods, and their distribution through the plant, the methods used for estimating these changes must be described in some detail. Since cacao seedlings, like the mature trees, are highly variable, comparison of the uncorrected weights of successive samples was considered to give insufficient precision for the purpose in hand, and methods were sought for providing more accurate estimates of the changes taking place. The principle adopted in most instances was to use the weights and linear measurements of the plants forming one harvest to establish a relationship between these quantities, and then to use measurements made at the same time on plants harvested subsequently to estimate the weight for each of these plants at the time of the first harvest. The difference between this estimate of the initial weight and the weight actually determined 6 weeks later then provided the estimate of the change in weight during that period. This technique was essentially the same as that used for estimating dry-weight changes in tomatoes (Goodall, 1945), though in that case the periods over which changes were measured were of a few hours only. In the present study, as in that with tomatoes, several possible ways of using the preliminary measurements for estimating initial weights were tested, and those selected as giving the lowest errors of estimation are detailed below:

Dry weight. The dry weight at planting of the seedlings harvested at 6 weeks was estimated from the fresh weight of the seeds, together with their mean percentage dry weight. The mean proportions of radicle and cotyledons in other series of observations (Goodall, 1949) were taken as applicable.

Estimates of dry weight of leaves and stems at 6 weeks and subsequently were based on their linear dimensions on the days preceding harvests (or on the last measurement before that date, if the leaves were not then growing). For *mature leaves* the ratio of dry weight to product of length and breadth on the day preceding harvest was found to be independent of leaf size, and thus the weight of a mature leaf at the beginning of an experimental period was estimated from the product of its length and maximum breadth, together with the mean value of this ratio for plants harvested at that time. For *immature leaves*, which at any harvest were relatively few in number, the relationship between dry weight and the product of length and maximum breadth, though close, could not be fitted satisfactorily by a simple mathematical expression, so freehand curves were drawn connecting these values at each harvest, and the lengths and maximum breadths of immature leaves of the subsequent harvest were used to read from these curves their estimated initial dry weight. The dry weights of *stems* were estimated from a linear regression of log. dry weight on log. length. In the case of the *cotyledons* it was found that the proportion of dry matter lost by them during the 6 weeks following germination was related to the total amount of foliage-leaf material at the end of this period; accordingly the regression of the ratio of cotyledon dry weight at 6 weeks to seed fresh weight at planting on the sum of the products of leaf length and maximum breadth at 6 weeks was used to estimate the dry weight at that time of cotyledons subsequently lost by abscission. A linear regression of *root* dry weight at 6 weeks on seed fresh weight at planting was used to estimate the dry weight at that time of roots of plants harvested at 12 weeks. At 12 weeks and subsequently root dry weight could more accurately be estimated from a regression of log. root dry weight on log. stem length.

The dry weight of the *whole plant* was on each occasion estimated as the sum of the estimates of dry weight of the individual organs.

Leaf area. In view of the difficulty of measuring the areas of large numbers of small leaves while still attached to the plant, and the risk of damage, area measurements were made only on leaves separated from the plants at harvest-time. Areas of leaves of plants not harvested were estimated from a linear regression of area on the product of length and maximum breadth.

Water weight. The weights of water in individual *leaves* at 6 weeks and subsequently were estimated from linear regressions of weight of water on product of length and maximum breadth, those in *stems* from linear regressions of log. weight of water on log. stem length. At 6 and 12 weeks the weight of water in the *roots* was estimated from linear regressions of the values for plants harvested at those times on seed fresh weight at planting; at 18 and 24 weeks a more accurate estimate of the weight of water in the roots was given by a linear regression on the sum of the products of length and maximum breadth for each leaf of the plant. In the *cotyledons* the weight of water at 6 weeks was likewise estimated from a linear regression on the sum of products of leaf length and maximum breadth.

As in the case of dry weight, the weight of water in the *whole plant* was estimated by summing the estimates for the various organs.

Errors of estimation. The errors involved in the methods of estimation described were found by using them to estimate the 'changes' between duplicate samples harvested simultaneously. In the 6-week and 12-week harvests alternate plants were used for the determination of parameters, which were then used for estimating the 'changes' in dry weight and water weight of the organs of the other twenty plants in the same harvest. In Table I are shown the root mean squares of the 'changes' thus determined, together with the mean weights of the organs:

TABLE I

Errors of Estimation of Changes in the Weight of a Plant and its Parts

		6 weeks.		12 weeks.	
		Observed mean (mg.).	Root mean square deviation.	Observed mean (mg.).	Root mean square deviation.
Dry matter:	Leaves	215	20	607	72
	Stem	50	7	159	43
	Roots	275	28	515	80
	Cotyledons	596	56	—	—
	Whole plant	1,136	54	1,281	153
Water:	Leaves	614	71	1,193	117
	Stem	235	27	538	97
	Roots	1,602	195	2,130	337
	Cotyledons	1,361	200	—	—
	Whole plant	3,812	301	3,861	373

It will be seen that in most cases the error of estimation for organs of a single plant is between 10 and 15 per cent. of the mean weight. Thus, for a sample of forty plants harvested, the errors of estimation of the mean changes of weight were of the order of 2 per cent. of the initial weight.

As examples of the increase in precision with which changes are estimated by this procedure, Table II shows the changes in weight of water in the plant and its organs between 6 and 12 weeks from planting, as estimated by the methods described, and also by simple comparison of the means of successive samples without taking the concomitant measurements into account. The considerable reduction in the standard errors for leaves and stem is clear. In this particular set of data there was no improvement in precision in the estimate for the cotyledons, and that for the roots did not reach significance;

nevertheless the change in the plant as a whole was estimated much more precisely when the concomitant measurements were used.

In Table II, as also in subsequent tables, variance between progenies has been eliminated from the error estimates. Except where explicitly mentioned it proved to be non-significant. The figures quoted for error between progeny means do not, of course, include any contribution on account of the errors in the parameters used for the estimations—the same parameters having been used for all progenies; the error estimates appropriate to comparisons between different harvests are consequently somewhat greater—usually by no more than 5 per cent.—and the necessary adjustments have been made in determining the significance levels quoted in the text.

TABLE II

Changes in Weight of Water in Plant between 6 and 12 Weeks Estimated by Two Methods

	Without use of concomitant measurements.		With use of concomitant measurements.	
	Mean estimate of change (mg.).	S.E. of mean.	Mean estimate of change (mg.).	S.E. of mean.
Leaves . .	+ 453·4	72·2	+ 581·2	41·8
Stems . . .	+ 231·9	37·2	+ 266·5	20·5
Roots . . .	+ 570·3	56·9	+ 604·7	49·9
Cotyledons . .	− 19·8	37·6	− 62·5	41·9
Whole plant (incl. cotyledons) .	+1235·8	133·1	+1389·9	100·2

RESULTS

Dry weight

The main features of the dry-weight changes are presented in Fig. 1. The numerical data used in preparing this diagram are composite—that is, the values shown for the harvest at 6 weeks are the actual means for these plants, and those at planting the estimated initial values for the same plants; the values on subsequent occasions, however, were obtained by adding the estimated increases during each period to the actual weights of the respective organs harvested at 6 weeks. The weights of organs lost by abscission are shown in the narrow intermediate columns; these losses might occur, of course, at any time during the 6 weeks separating successive harvests. The cotyledons, which form the major part of these losses, were, on average, shed 68 days after planting.

Over the first 12 weeks of growth the net increase in plant dry weight is negligible—that is, the gain in weight of the whole plant including cotyledons is little more than the weight of the latter at the time of abscission. Thereafter, however, the gain in dry matter is approximately exponential, the dry weight having nearly doubled after each interval of 6 weeks.

In Table III are given the mean relative growth rates for plants of each parentage over each period. For the period 6–12 weeks the dry weight of the cotyledons at abscission has been included in the final plant dry weight.

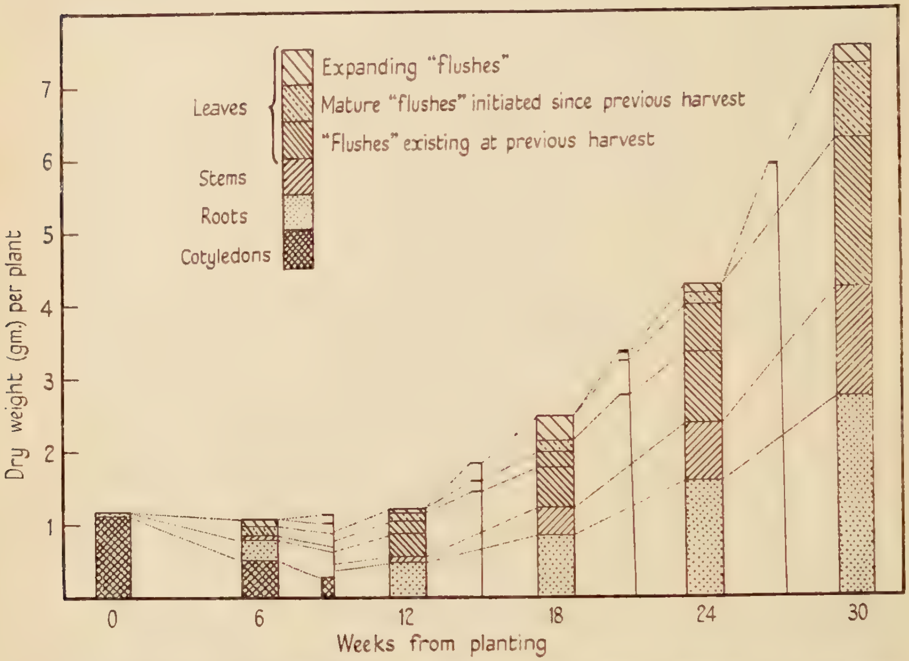

FIG. 1. Increase in dry weight of the cacao seedling and its parts during the first 30 weeks after planting. The narrow intermediate columns represent organs shed during the inter-harvest periods.

TABLE III

Mean Relative Growth Rates (% per day)

Female parent:	E54/122.	E62/129.	E62/31.	E61/93.	Mean.
6–12 weeks . . .	0·71	0·73	0·80	0·64	0·72
12–18 weeks . . .	1·28	1·79	2·07	1·93	1·77
18–24 weeks . . .	1·03	1·28	1·40	1·52	1·31
24–30 weeks . . .	1·04	1·05	1·42	1·85	1·34
Mean, 6–30 weeks . .	1·02	1·21	1·42	1·49	1·29

S.E. of progeny means, 24–30 weeks: 0·14
,, ,, other periods: 0·10

The differences from period to period are significant, even if the first period is excluded. It is clear that the low values of 6–12 weeks are in part due to the inclusion of some inert material in the cotyledons, later shed. Whether the high values for 12–18 weeks are a special characteristic of this stage of development, or whether they are fortuitous, associated perhaps with the relationship of harvest dates to 'flushing', cannot be determined without a more extensive series of data. The differences between progenies and their interaction with period are also significant. Though these differences are most probably of genetical origin, that is not necessarily so; only two pods from each parent having been used, it is, for instance, possible that differences in ripeness of these pods might account for them.

The proportional distribution of the increase in dry matter among the various organs of the plant differs substantially from period to period, as is clear from Table IV.

TABLE IV

Mean Percentage Distribution among Organs of Increase in Dry Matter of Whole Plant excluding Cotyledons

	Roots.		Stem.		Leaves of mature ‘flushes’.		Leaves of expanding ‘flushes’.		Leaves of new ‘flushes’.	
Period.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.
0–6 weeks	52·6	3·1	9·2	0·3	—	—	—	—	38·2	2·8
6–12 weeks	31·0	1·3	13·5	0·7	7·8	1·6	15·8	3·8	31·9	2·0
12–18 weeks	29·4	1·5	17·4	0·7	3·1	0·6	13·2	3·5	36·9	3·5
18–24 weeks	42·9	2·4	20·8	0·9	3·2	0·6	16·4	2·5	16·7	3·0
24–30 weeks	32·4	3·3	19·4	1·7	1·1	0·6	2·0	0·4	45·1	4·7

In Table IV by ‘new flushes’ is meant those of which the expansion began after the commencement of the period in question; the terms ‘expanding’ and ‘mature’ likewise refer to the condition of the ‘flushes’ in question at the beginning of the period. The error estimates quoted in Table IV are the root mean square deviations of the percentages for each individual plant from the mean for the progeny of which it is a member; since these individual data are quotients of two variables, and hence will have a non-uniform error component, the error estimates should be taken as no more than a rough indication of the variability of the results.

The principal progressive changes shown in the data are an increase during the first four periods in the proportion of new material laid down in the stem and a decrease in the proportion remaining in the mature leaves. During the fourth period the proportion of new material laid down in the roots was particularly high, that in the leaves of new ‘flushes’ exceptionally low; the number of new ‘flushes’ formed during this period was lower than in any other (0·7 per plant), and the high proportionate figure for root increase suggests that root growth may follow a cycle in opposite phase to that of leaf development.

Of the increase in dry weight of the whole plant excluding the cotyledons between 6 and 12 weeks, a substantial part may be accounted for by translocation from the cotyledons, which lost 256 mg. of dry matter between 6 weeks and the time when they were shed. Even if the cotyledons were losing dry matter by respiration during this period at the maximum rate (1 per cent. per day) recorded previously for entire seedlings (Goodall, 1949)—an allowance which seems excessive unless they had at this stage entered a marked climacteric—the amount translocated would still represent 21·4 per cent. of the net gain by the rest of the plant between 6 and 12 weeks, and of course a substantially higher proportion of the gain during the 26 days for which the cotyledons remained attached to the plant.

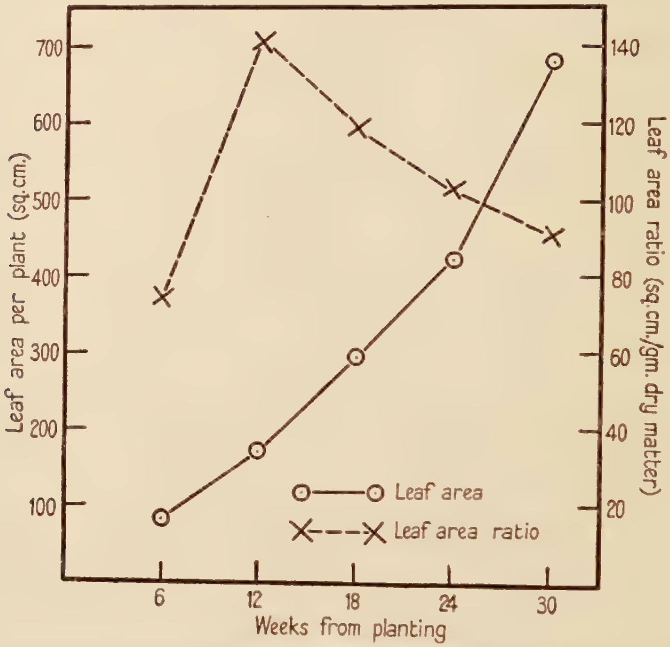

FIG. 2. Changes in the total leaf area per plant and in the ratio of this to the plant dry weight during the first 50 weeks of the development of cacao seedlings.

Leaf area

The development of total leaf area is shown in Fig. 2, sampling errors having been adjusted in the same way as for the dry-weight data in Fig. 1, the 12-week harvest being taken as the basis for adjustment. Table V shows the mean rate of increase in leaf area for plants of each parentage over each period from 6 to 30 weeks. In both Fig. 2 and Table V the area of the cotyledons has been neglected.

TABLE V

Mean Rates of Increase in Leaf Area (*sq. cm. per week*)

Female parent:	E54/122.	E62/129.	E62/31.	E61/93.	Mean.	S.E. of progeny means.
6–12 weeks . . .	13·4	17·8	17·0	14·1	15·6	1·7
12–18 weeks . . .	13·9	19·0	20·7	24·5	19·5	3·0
18–24 weeks . . .	17·0	19·5	23·1	33·3	23·2	6·6
24–30 weeks . . .	35·3	33·1	39·7	56·9	41·3	10·8
Mean, 6–30 weeks . .	19·9	22·4	25·1	32·2	24·9	3·3

The rate of growth in leaf area increased progressively during the experiment; the average rate over all periods was significantly greater for the progeny of E61/93 than for the others.

The leaf-area data provide the opportunity for estimating the net assimilation rate of these seedlings. The usual formula for this, as Williams (1946) has pointed out, involves the assumption that changes in leaf area and in plant dry weight are linearly correlated; in a plant like cacao where leaf-area development is irregular it is clear that this assumption is hardly justified. Whereas leaf-area increase in an individual plant follows a step-wise course, that in plant dry matter, apart from diurnal fluctuations, is more nearly continuous, with probably a slight decline in the rate during the periods of most rapid leaf expansion. Consequently some plants might form a new 'flush' and perhaps double their leaf area shortly after the beginning of an experimental period, in which case the usual formula would overestimate the mean net assimilation rate, while in other plants a similar leaf expansion might occur immediately prior to the end of the experimental period and result in an underestimate of the mean net assimilation rate.

The true mean value of the expression $\frac{1}{L}\frac{dW}{dt}$ (L being leaf area, W plant dry weight) over the period studied cannot be found unless the course of increase in both leaf area and plant dry weight is known in detail. In the present work, daily leaf measurements convertible into area estimates were available, but nothing was known of the course of increase in plant dry weight between harvests. In an early paper Gregory (1926) suggested a method of calculating net assimilation rate involving repeated leaf-area measurements and an assumption of linear increase in plant dry weight; it appeared that this assumption would not be far from the truth over a period of 6 weeks in a slowly growing plant like cacao. Accordingly the net assimilation rate for each plant harvested was computed for the preceding period of 6 weeks by two methods: (*a*) the usual formula, including initial and final leaf areas and plant dry weights, involving the assumption of linear relationship between the two variables; and (*b*) Gregory's suggested method, using daily leaf-area estimates with initial and final plant dry weights, involving the assumption of linear increase in plant dry weight. The means computed by both methods are shown in Table VI. Separate estimates having been made for each plant, the standard errors shown have been derived from the variance among these individual estimates. For the period 6–12 weeks, as in determinations of relative growth rate, the dry weight of the cotyledons at abscission had been included in the final plant dry weight.

The error variance of the estimates is greater over the first period (6–12 weeks) than for subsequent periods when computed by method (*a*), but not by method (*b*); furthermore, the error variance is significantly greater using method (*a*) than using method (*b*) up to 18 weeks; both these facts suggest that method (*b*) gives, in this case, more reliable and consistent results than method (*a*). Between 6 and 12 weeks the mean net assimilation rate is

TABLE VI

Mean Net Assimilation Rates of Cacao Seedlings (g./sq. dm./week), computed by Two Methods

Method of computation (see text).	Female parent: Period from planting.	E54/122.	E62/129.	E62/31.	E61/93.	Mean.	S.E. of progeny means.
(*a*)	6–12 weeks	0·0484	0·0798	0·0730	0·0496	0·0627	0·0104
	12–18 weeks	0·0562	0·0830	0·0909	0·0832	0·0783	0·0051
	18–24 weeks	0·0620	0·0691	0·0771	0·0790	0·0718	0·0056
	24–30 weeks	0·0656	0·0709	0·0920	0·1246	0·0883	0·0079
	Mean, 6–30 weeks	0·0581	0·0757	0·0833	0·0841	0·0753	0·0037
(*b*)	6–12 weeks	0·0480	0·0612	0·0593	0·0463	0·0537	0·0058
	12–18 weeks	0·0544	0·0740	0·0799	0·0744	0·0707	0·0037
	18–24 weeks	0·0589	0·0668	0·0784	0·0782	0·0706	0·0048
	24–30 weeks	0·0706	0·0776	0·0962	0·1276	0·0930	0·0081
	Mean, 6–30 weeks	0·0580	0·0699	0·0785	0·0816	0·0720	0·0028

estimated 16·8 per cent. higher by method (*a*) than by method (*b*), and between 12 and 18 weeks 10·4 per cent. higher, while thereafter the differences are negligible. The fact that the discrepancies between the two methods diminish as development proceeds may probably be explained as follows: the proportion in which each new 'flush' increases the pre-existing leaf area of the plant diminishes as the plant grows and the number of 'flushes' it bears becomes greater; consequently, at earlier stages, individual variation in the time of 'flush' expansion within the experimental period would affect the values obtained by method (*a*) more markedly than at later stages.

In the previous study covering the first 7 weeks of growth of cacao seedlings (Goodall, 1949) the maximum net assimilation rate was estimated at 0·090 g./sq. dm./week, and comment was made on the lowness of this value compared with data for other crop plants in the literature. The present results fully bear out this conclusion as to the low net assimilation rate of seedling cacao, but suggest that it was increasing as development proceeded, particularly in the progeny of E61/93. Time and progeny differences and their interaction were all highly significant.

The broken line in Fig. 2 represents the ratio of mean leaf area to mean plant dry weight at each occasion of harvest. It will be noted that this ratio reached a maximum at about 12 weeks, and thereafter declined. The true position of this maximum, had more frequent observations been made, might well have been found to coincide with the time at which expansion of the first 'flush' of leaves was complete, between the 6-week and 12-week harvests. It will be noted, however, that the greatest mean inter-harvest value of this ratio occurred during the period (12–18 weeks) when the greatest relative growth rates were recorded, and may well have been responsible for this.

Water-content

In Fig. 3 the absolute weights of water in the plants and their organs are presented in the same fashion as the dry-matter data in Fig. 1. The diagram speaks for itself, and little comment is necessary. The general picture is very similar to that for dry matter, with the exception of the first 6 weeks after planting, during which the water-content increased by a factor of 9 while the dry weight remained almost unaltered. A detailed study of this period of development has already been published (Goodall, 1949).

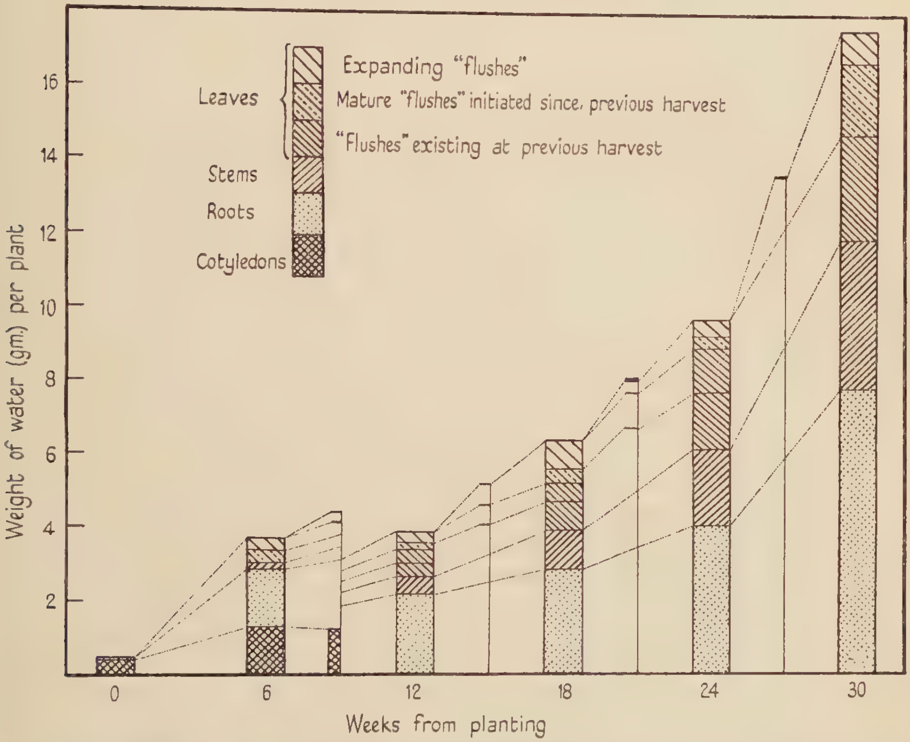

FIG. 3. Increase in weight of water in the cacao seedling and its parts during the first 30 weeks after planting. The narrow intermediate columns represent organs shed during the inter-harvest periods.

In Table VII are shown the proportions in which the net gain in water by the plant as a whole was distributed among the various plant organs; the comments on the standard errors quoted in Table IV are equally applicable here. The cotyledons are not included in Table VII; between planting and the first harvest, the net increase in water-content of the cotyledons amounted to 37·4 per cent. of that in the remainder of the plant, and at 6 weeks they contained 1,282 mg. water per plant. Thereafter there was a decrease in water-content to abscission amounting on average to 62 mg. per plant. By 6 weeks after planting senescence of the cotyledons is already far advanced.

The rate of gain in water-content in the roots as a percentage of that in the

Table VII

Mean Percentage Distribution among Organs of Net Increase in Absolute Water-content of Whole Plant excluding Cotyledons

	Roots.		Stem.		Leaves of mature 'flushes'.		Leaves of expanding 'flushes'.		Leaves of new 'flushes'.	
Period.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.
0–6 weeks	64·4	2·0	9·9	0·4	—	—	—	—	25·7	1·7
6–12 weeks	41·1	1·7	18·2	1·0	−1·4	0·9	5·7	1·3	36·3	1·7
12–18 weeks	24·7	2·2	23·5	1·7	−4·3	1·9	11·8	3·6	44·3	4·7
18–24 weeks	43·4	4·2	27·3	2·7	−7·0	1·6	14·0	2·7	22·0	3·6
24–30 weeks	49·2	5·0	18·6	5·5	−9·5	2·7	—	—	41·7	4·4

whole plant decreased from germination (at one period of which practically the whole increase in water-content is in the radicle; Goodall, 1949) for about 3 months, and then increased to a value of about 45 per cent. The corresponding figures for the stem increased over the same period, and then remained approximately constant at about 25 per cent. Those for the leaves as a whole increased to a maximum of about 50 per cent. at 3 months, when the leaf-area ratio was at its highest and the relative rate of increase in leaf area also high, and then declined to about 30 per cent. No increases occurred in the weight of water contained in the leaves of mature 'flushes'; indeed, it was decreasing at all times, the rate of decrease in proportion to the increase in the plant as a whole becoming progressively greater as the experiment proceeded and the average age of these leaves increased. The varying distribution of the net gain in water between 'flushes' existing and expanding at the commencement of the 6-week period and those beginning expansion subsequently is probably largely fortuitous, depending upon the coincidence of leaf expansion with time of harvest. For instance, the fact that many 'flushes' were expanding at 18 weeks, few at 24 weeks, and many again at 30 weeks led to a much greater gain in water by new 'flushes' between 24 and 30 weeks than during the previous 6-week period. In the data from which Table VI has been compiled certain significant differences among progenies were found, but they were limited to the first two periods and do not seem to merit further comment.

In Table VIII and Fig. 4 are shown the water-content of the plants and their various organs expressed in terms of dry matter. After the initial rise in the water-content of the plant during the first 6 weeks, it fell steadily to 24 weeks, after which there was a slight but significant rise to 30 weeks. The water-content of roots and stems showed similar changes. Before 24 weeks the water-content of the roots was consistently higher than that of the stems, but in the last two harvests the values for the two organs were very similar. The water-content of the mature 'flushes' was lower than that of the other organs, and declined consistently through the experiment as the average age of the leaves included in them increased; that of the expanding 'flushes' was consistently high, except at 18 weeks, the low value for which may probably be accounted for by

TABLE VIII

Mean Water-content of Plants and Organs, Expressed as Percentage of Dry Weight

Time from planting.	Whole plant.		Roots.		Stem.		Leaves of mature 'flushes'.		Leaves of new 'flushes'.	
	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.	Mean.	S.E. of mean.
6 weeks	349	7	559	13	470	10	240	6	376	15
12 weeks	328	8	452	12	359	6	183	4	357	33
18 weeks	266	5	372	9	317	7	172	5	239	9
24 weeks	232	5	276	7	273	8	168	3	410	17
30 weeks	249	5	311	9	307	10	158	6	385	34

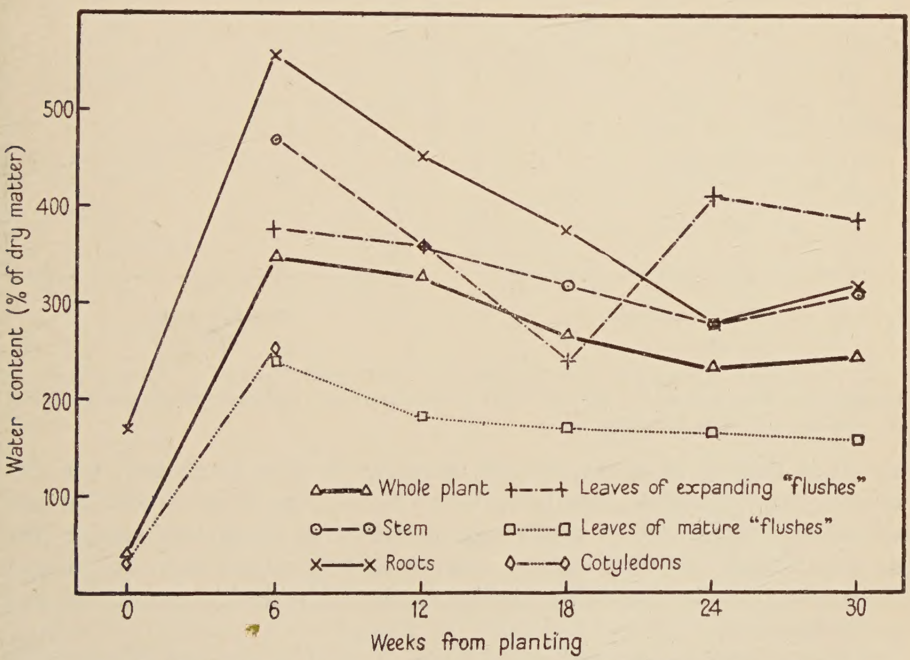

FIG. 4. Changes in the water-content (% of dry matter) of the cacao seedling and its parts during the first 30 weeks of development.

the fact that at this harvest, unlike others, the majority of the leaves in these flushes had completed expansion and were already 'hardening'. The mean percentage water-content of the cotyledons at the first harvest was 256 per cent. (S.E. = 6 per cent.). Parentage affected the percentage water-content of the whole plant, roots, and stem at 30 weeks, and that of the cotyledons at 6 weeks.

In the previous paragraph, attention was drawn to the progressive decline in percentage water-content in the leaves of mature 'flushes'. When data for the individual 'flushes' were separated, a similar decline in each was apparent.

This is shown in Fig. 5 *a*; although leaf expansion had ceased in these 'flushes', the percentage water-content in each continued to decline throughout the experiment, and at all occasions of sampling there was a progressive decrease in water-content from the younger to the older leaves. In the first, third, and fourth 'flushes' the change in percentage water-content during the experiment was adequately accounted for by a similar fall in the absolute water-content (Fig. 5 *b*). In the second 'flush' the mean absolute water-content was less at

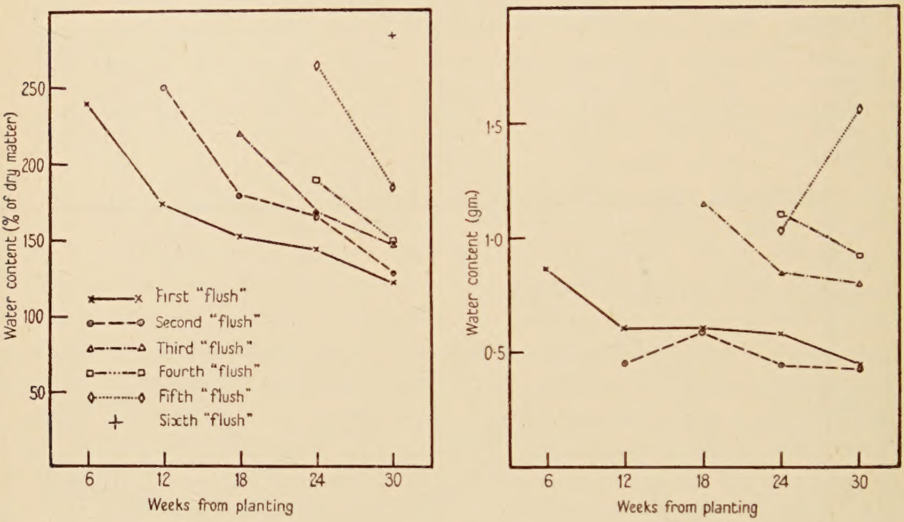

FIG. 5 *a*.

FIG. 5 *b*.

FIG. 5 *a* and 5 *b*. Changes in the water-content of successive 'flushes' of mature leaves in the cacao seedling during the first 30 weeks of development, expressed (*a*) as a percentage of dry matter, and (*b*) in grammes.

12 weeks than at 18 weeks, though thereafter it again decreased; this discrepancy may, however, have been due to the fact that those second 'flushes' which were mature at 12 weeks were probably not a random sample (the smaller 'flushes' maturing earlier), whereas those of plants harvested at later dates were; similar reasoning applies to the fifth 'flush' between 24 and 30 weeks.

Water-content data for individual leaves of 'flushes' expanding at the time of harvest are presented in Fig. 6. The relative rate of increase in area (over the period from 48 to 24 hours before harvest—the latest for which information was available) has been taken as an index of the stage of development; this remains constant at about 60 per cent. per day until the leaf reaches half its final area, and then declines sharply—90 per cent. of the final area being attained between 2 and 3 days later. The points in Fig. 6, though very scattered (the error of estimation of the relative rate of leaf-area increase was very high for small leaves), fall clearly around a curve which is asymptotic to a water-content of about 566 per cent. for leaves expanding rapidly, and meets the water-content axis at about 380 per cent. for leaves whose expansion ended one day earlier. It would appear that the leaf water-content begins to fall as soon as the relative rate of leaf expansion decreases, and continues to fall after the leaf, and the 'flush' in which it occurs, have ceased expansion; the mean water-content of fully expanded leaves of 'flushes' of which younger leaves were still expanding was 347 per cent., which may be compared with the values for mature 'flushes' in Table VIII.

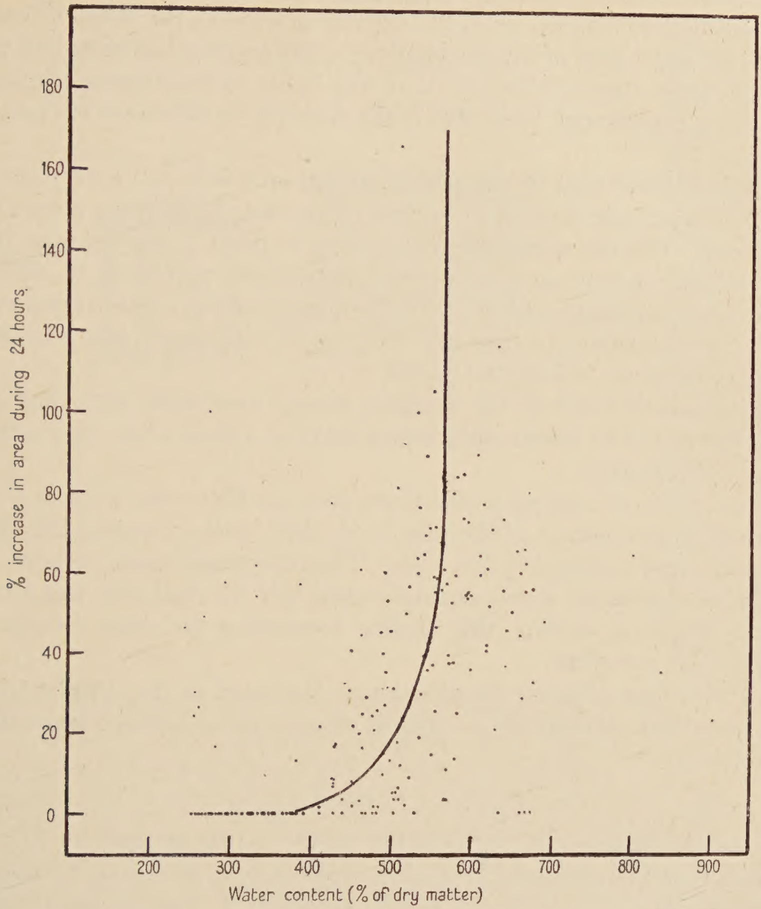

FIG. 6. The relation between the water-content (% of dry matter) of leaves of expanding 'flushes' and their stage of development (as indicated by relative increase in area between 48 and 24 hours prior to harvest).

SUMMARY

The growth of cacao during the first 30 weeks after planting was studied by taking harvests of seedlings at intervals of 6 weeks. The fresh weight and dry weight of each organ individually was determined, and also the area of each leaf. Diagrams are presented showing the mean distribution of dry matter and water between organs at each harvest. Estimates of changes in weight of the plant and its parts were improved by measuring the intact plants before harvest and correcting for differences in these measurements.

The mean relative growth rate of all plants between 6 weeks and 30 weeks from planting was 1·29 per cent. per day; it varied with the stage of development. The major part of the gain in dry matter occurred in roots and young leaves; the proportion of this gain occurring in the stems increased during the period of the experiment, while that in the older leaves decreased but remained positive.

Though in individual plants growth in leaf area followed a very irregular course, the mean rate showed a progressive increase through the period of the experiment. The net assimilation rate of each plant was computed by the usual method, and by another method (considered preferable in this case) involving daily estimates of leaf area. The mean net assimilation rate between 6 and 30 weeks from planting was 0·072 g./sq. dm./week, but it showed a tendency to increase during the period.

Distribution of water in the seedling agreed in general with that of dry matter, but the older leaves were losing water at a time when they were still gaining in dry matter.

Between 6 weeks and 24 weeks from planting there was a decline in the percentage water-content of the plant and of its various organs, this decline being particularly marked in the roots. The percentage water-content of the young leaves remained nearly constant until half the final area was attained, and then began to decline, this decline continuing for some months after expansion was complete.

Seed from four different female parents was used in the experiment, and it was found that certain of the growth characteristics studied were affected by this factor.

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